PARASITOLOGY

(Founded by G. H. F. Nuttall, F.R.S.)

EDITED BY

D. KEILIN and P. TATE

IN CONJUNCTION WITH

A. BISHOP
F. J. BROWN
P. A. BUXTON
E. HINDLE
C. A. HOARE
G. LAPAGE
K. M. SMITH

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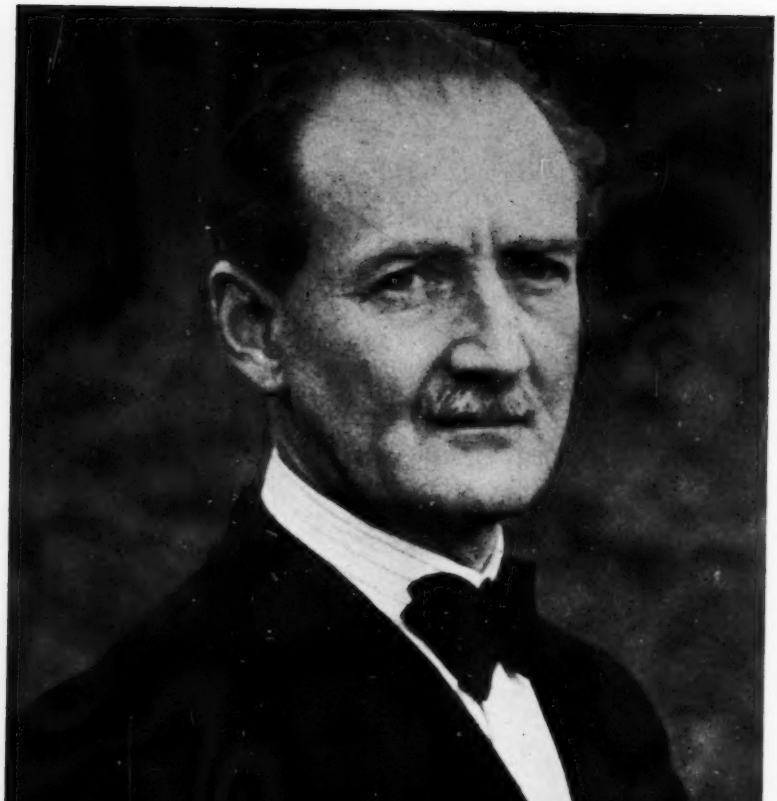

CLIFFORD DOBELL (1886–1949)

VOLUME 42, NOS. 1, 2 MARCH 1952

CLIFFORD DOBELL (1886–1949)

IN MEMORIAM

BY DORIS L. MACKINNON* AND CECIL A. HOARE†

When, on 23 December 1949, Clifford Dobell died as the result of an unheralded cerebral haemorrhage, the world of biological science lost a remarkable figure and this *Journal* one of the most distinguished of its regular contributors. Dobell's name means much to protistologists all over the world, especially to students of the parasitic Protozoa, who recognize in him a great investigator of the animal microcosm. His contributions to the history of his science, culminating in the famous monograph on Leeuwenhoek, reveal him also as a man of wide culture and endowed with a sense of the past such as is rare in these days.

Cecil Clifford Dobell was born on 22 February 1886 at Birkenhead. He was the elder son of William Blount Dobell (1859–1927), who was first a coal-merchant in Birkenhead and then the owner of a colliery in South Wales. In physique, if in little else, Clifford took after his father, but in character and tastes he derived more from his mother, Agnes Thornely (1852–1942). Agnes was brought up by her grandfather in Liverpool, where she was given a more liberal education than was usual for a girl of her generation. She was an excellent linguist and an accomplished musician. Dobell undoubtedly inherited from her his linguistic gift and a passionate love of music: also from her came the strong, tenacious will that carried him through a difficult life.

A capacity for business certainly was not one of William Dobell's gifts, and, as Agnes had no money sense either, frequent fluctuations in the family fortunes considerably affected Clifford's boyhood. Between the ages of six and ten, when things were going satisfactorily, the Dobell children lived a free life in beautiful country not far from their father's colliery at Llandebie. It was here that Clifford acquired his taste for natural history and had the chance to keep pet animals to his heart's content. More than most children of his age, he was interested in 'insides' and he began to dissect dead animals with much curiosity. His manipulative skill revealed itself very early in a variety of ways: he quickly learnt carpentry, and showed great promise with pencil and brush. He read avidly all such books as came his way: but, until he was ten, his education, beyond the kindergarten stage, was left to an inadequate governess. After four years of an idyllic existence in South Wales, things deteriorated at the colliery, and the children were condemned to spend the next three years in 'rooms' at Southport. Then money became easier again, and life in the country was once more possible. But by this time Clifford was thirteen, and he was left behind at Southport as a boarder at Sandringham School.

It was only now that he was subjected to formal education. During the last three years, it is true, he had been attending classes in French, swimming, gymnastics and—to his great delight and benefit—drawing, modelling and painting at the Southport School of Art. But settling down to the ordinary school curriculum did not come easy, and he was not the sort of boy to take naturally to communal life. Moreover, although strong and active, he had little interest in organized games. It was fortunate for him, therefore, that his headmaster, Mr Chadwick, promptly recognized the unusual quality of his new pupil and the benefit he had gained from wide and varied readings of his own choice. Clifford quickly responded to the sympathetic help he got, and set himself to justify it by working hard and maintaining even in unfamiliar studies his place at the top of his class. By the time he was sixteen he was doing special work with Mr Chadwick himself, who gave him an excellent grounding, not only in the classics but also in mathematics and chemistry. For the last eighteen months at Sandringham School he was given his head completely and was allowed to work at just what he chose. This proved quite a safe line to take with one so passionately curious and so many-sided in his interests. On his own account, it would seem, he maintained an educational balance by much reading and memorizing of a wide range of English poetry and by taking up a study of comparative religion that presently decided him to refuse Confirmation. Although he continued to draw and paint, he had long since given up a childish ambition—with which his father had surprisingly sympathized until discouraged by Briton Riviere, R.A., who said there was no money in professional art—to become a painter of animals; and he was increasingly drawn to a career in which biological science should play a part. Chadwick urged strongly that his favourite pupil, young though he still was, should now go to Cambridge; and, since the Dobell family fortunes were once more in decline, he himself generously

* From University College, London.

† From Wellcome Laboratories of Tropical Medicine, London.

advanced the money that would make this possible. It was not until 1918 that Clifford discovered that this debt had not been repaid; he at once settled it—and with compound interest—out of his small savings. In considering Dobell's difficulties, it is well to remember that he very early found himself responsible for his father's debts and for the financial support of his mother—who lived to be ninety. To her he was devoted, and nothing he could do was too much for him to offer willingly; but as she insisted on living in a remote district of Wales, and for the most part quite alone, he had not only to keep up two establishments but also was constantly harassed—especially in her old age—by anxiety on her account.

Dobell entered Trinity College at the age of seventeen. Since he still 'preferred animals to people', his first intention, on going to Cambridge, was to become a veterinary surgeon. What then deflected him to the study of medicine is not known. But it is quite certain that the advice of his tutor at Trinity, Adam Sedgwick, was responsible for his deserting medicine for pure science after he had passed his Second M.B. Examination with distinction in anatomy and physiology. By offering zoology and chemistry, in addition to the medical subjects, Dobell gained his B.A. degree at the Natural Sciences Tripos in 1906 with First-Class Honours and the highest marks for the whole University. His contemporaries have thought it 'a pity' that he never proceeded to Part II of the Tripos; but undoubtedly money was the determining factor: he was living on borrowed money, and the sooner he could earn something, the better for his pride. In 1905 he had gained an Exhibition in Natural Sciences, and now he was awarded a Major Scholarship. From this time onwards, he was to be a professional zoologist. He took his M.A. degree in 1910. He might have had his Sc.D. at any time after 1918, but it was not until 1942 that he sent in his application for the doctorate; even then it was with reluctance and, as he put it, 'only for business reasons'.

Dobell always accounted himself extremely fortunate to have come under Sedgwick's influence, and he could never say enough in expression of his gratitude for the advice, help and encouragement he got from that remarkable man—'my father in zoology, and my best friend till the day of his death' (cf. Dobell, 1949). During the first four years of Dobell's residence at Cambridge, the ornithologist Alfred Newton, first occupant of the Chair of Zoology and Animal Morphology, was still ostensibly the head of the Department, but it was Adam Sedgwick who 'delivered all the professorial lectures and directed the Zoological Laboratory in another part of the old buildings'. Sedgwick, who had been appointed Reader in Animal Morphology in 1882, 'remained in this nominally subordinate position, professor in all but name and pay . . . till Newton's death in 1907' (loc. cit.).

Sedgwick dreamed of developing a Department of Protozoology at Cambridge, and it was he who directed Dobell's interest towards this study. As a beginning, he had had W. S. Perrin trained abroad in the recent techniques, and now he arranged that Dobell should collaborate with Perrin and make a start with research. For the best part of the next two years these young men, united by a common enthusiasm and much personal liking, worked happily side by side in a dingy little room 'for seven days in every week and for anything up to sixteen or seventeen hours a day. All the physical recreation we could ever afford was an occasional game of tennis or a swim in the river' (1936*a*). They lived on their scholarships and by what they could pick up by coaching in the evenings. Dobell continued to attend advanced courses in zoology, physiology, biochemistry and bacteriology. Nevertheless, he found time and energy to play an active part in the Cambridge Natural Sciences Club, of which he took his turn as secretary and as president, and to which he read a number of papers on such unrelated subjects as parthenogenesis, death, phrenology, the future of man; and, characteristically, one in mockery of Driesch's entelechy—a theory fashionable at that time among biologists.

While at Cambridge, in matters of arts and letters Dobell was much under the sway of the later nineteenth century. The influence of Aubrey Beardsley, Max Beerbohm, and the Japanese is clear in his drawings at this time; and we are told that he lined his living-room with black wall-paper on which were hung reproductions of Beardsley's 'Salomé'. His 'light literature' was represented by Maupassant, Poe, Wilde, Ibsen and Shaw, while his philosophy was pessimistic—he was a great admirer of Schopenhauer and Leopardi. Music was always a vital necessity for him. There his tastes were all for the Romantics; for Chopin especially he had a feeling that was near idolatry, and, while many of the other youthful enthusiasms were transient, this Chopin fixation remained with him through life. Although he could play the piano by ear, he had never had time to master the instrument as his mother had when young; so, as soon as he could afford it (and that was not until he was a Fellow), he bought the pianola that was one of his treasured possessions for the rest of his days. It was characteristic that he tuned the thing himself and altered the faulty notes in the rolls.

Since Dobell never assimilated anything foreign to his own temperament, it is conceivable that in his twenties he was unconsciously selecting what at any time would most have appealed to him. A friend, commenting on Clifford's insistence that Chopin was the greatest musician of all time, remarks: 'Perfec-

tion in a circumscribed technique—such as composition for the piano—he held to be of greater value—higher in the scale of art—than incomplete perfection on the grander scale', and adds very astutely: 'I think this explains a good deal about Dobell.'

The same Cambridge contemporary writes of Dobell as he was at that time: 'Although he was a year or more younger than any of his set, he was easily pre-eminent among them, partly on account of his looks and partly because of his extremely ready (and rather sarcastic) tongue.' The looks were not of the conventional sort, but conveyed a sense of 'style'. He was of medium height and slight build, and carried well his fine head, with its aquiline profile; even at sixty he had lost nothing of his early air of distinction.* The rather unexpected effect that he produced on his undergraduate acquaintances was heightened by their admiration for what seemed to them his magical skill with pen and pencil. He could hit off a caricature likeness in a few telling lines, and his amazing visual memory enabled him to draw faces, months afterwards, that he had once seen in a crowd.

The friends he made he kept. But he did not get on close terms with many, for though he longed for sympathy and was immediately responsive to kindness and understanding, he was very shy: and he was too 'intense', too moody, and too much inclined to set for others his own standards of thought and conduct. The average young man found these alarming.

These standards of his youth remained practically unchanged throughout his life. His intellectual and moral probity were matched by the seeming austerity of his physical habits. Though not athletic in the accepted sense of the word, he attached great importance to bodily fitness. He swam or walked whenever the chance offered; he never wore hat or coat, whatever the weather; and up to the day before his fatal illness, he took his cold bath every morning. But this discipline was deliberately imposed. No one more knowledgeably appreciated what are called the good things of life—good food, good wine, well-cut clothes, a comfortable background. The fact was that he had expensive tastes, but could seldom afford to indulge them; and since, for him, nothing but the best would serve, he chose to do without, except when he had a guest—and he was very hospitable. In congenial company his gaiety and humour were something his friends will not forget, and the legend of Dobell as a bitter-tongued misanthrope is hard for them to accept.

At the end of 1907, having acquired all that Perrin could teach him of protozoological technique, Dobell went to Germany for further study. In the decade before the First World War, this was the accepted thing to do; Germany was regarded as the finishing school for British students of science, and the Germans had staked a claim in up-to-date protozoology. Schaudinn, Hartmann, Prowazek were all the vogue; Bütschli was, rightly, still a name to conjure with, and Richard von Hertwig, though getting on in years, still conducted a large research school in the University of Munich. It was to Munich that Dobell went, but the experiment was not a success. He admired Hertwig himself and, among the younger men, had good relations with Franz Doflein, whose work he found admirable and whose personality was very sympathetic to him—the more so, perhaps, as Doflein too had at one time toyed with the idea of 'going in for art'. But Dobell disliked the point of view of most of his fellow research workers. Hertwig himself appeared little among them in the laboratory, and most of the Germans there were so completely under the spell of Schaudinnian doctrine that Dobell felt stifled in their company. For a man who believed so passionately in 'studying things as they are, in preference to speculating on things which *must* be, or which *ought* to have happened', their working out of preconceived life histories and searching for non-existent structures were distasteful in the extreme. Almost the only benefit he got from his stay in Germany was its music. Concerts were unbelievably cheap, and, in company with an English fellow-student, he went to three or four a week; he also haunted the gallery of the opera house and had his first real experience of Wagner. But it was characteristic of him that his antipathy for the conditions then prevalent in German scientific circles made Germany as a whole something to detest for the rest of his life. Dobell delightedly quotes Leeuwenhoek as saying that he 'never trusted... Germans' (1932). But all this did not prevent his taking endless pains and spending money he could ill afford in helping a German zoologist, whom he particularly disliked, from the consequences of being stranded in London at the approach of war in the summer of 1914.

On leaving Munich, Dobell went to the Zoological Station at Naples, and here he felt very much more at ease. Unsupervised, his research made better progress. He began his investigation of the Sporozoa of cuttle-fish and crabs, and when he left, in June 1908, he had collected most of the material he was to use for his remarkable study of *Aggregata*.

When he returned to Cambridge he was elected a Fellow of Trinity College (1908–14). In that same year (1908) he won the Rolleston Prize (Oxford and Cambridge) and the Walsingham Medal (Cambridge) —both for original research in biology—and he was awarded the Balfour Studentship of Cambridge for 1908–9. This studentship enabled him to go abroad again—this time to Ceylon, where he worked on a variety of parasitic Protozoa at the laboratories

* The accompanying portrait (Plate I), which is in the 'Portrait Gallery' of parasitologists at the Molteno Institute, Cambridge, was taken in 1939.

of the Museum at Colombo and at the Botanic Gardens at Peradeniya. He received much hospitality and encouragement from Dr A. Willey, then Director of the Museum—'a fine naturalist and an honest man', he wrote in 1943 on hearing of Willey's death.

Except for an *Aggregata*-collecting stay at the Musée Océanographique at Monaco in 1913, and a brief visit to Holland in 1923 in connexion with his Leeuwenhoek studies, Dobell never again travelled abroad. For the rest of his life he was in England, and his fortnight's summer holiday in Cornwall was about all the respite he ever took from his imposed routine of a seven-day week in the laboratory.

In 1910 he left Cambridge to live in London, where Sedgwick, now Professor of Zoology at the Imperial College of Science and Technology and still bent on doing something to develop the study of protozoology in this country, offered his favourite pupil a lectureship (later assistant-professorship) in Protistology and Cytology. In a small Department, however, Dobell had much to do besides teaching his main subjects and supervising protistological research. With his usual thoroughness and honesty, he dutifully prepared himself for lectures and demonstrations, and his students found him a lucid and stimulating teacher. One of his former students, Dr Ernst Schwarz, writes: 'Dobell was entirely unafraid of "authority", always using his criticism freely and never leaving any doubt concerning his own views. This admirable method was far more stimulating than the stereotyped repetition of stale statements taken from current text-books.' Another associate from that time, Dr A. Pringle Jameson, thus characterizes him: 'All the qualities of the man were shown in his relations with those in his laboratory. He was, even in those days, a mine of information, and everyone was free to dig out all they wanted, but the digging had to be done, there was no handing out.... He was lavishly generous of his time and ideas, willing to hand on to others much of the mass of material which he always had "on tap"—cultures, work projects, and so forth....He never tried to take anything from those who worked with him—it was all give and no get. But if a "faker" tried to sponge on his goodness and slacked in his lab. he could be very cold.'

After Sedgwick's death, in 1913, he found less and less compensation for the expenditure of time and energy that teaching involves; he longed to devote himself to protistological research alone. So it was with alacrity that, in 1915, he accepted the invitation, through the Medical Research Committee and the Royal Society, to conduct, for the War Office, protozoological investigation of dysentery cases and carriers among the troops. Such teaching as he still had to do—for he was expected also to train other biologists in diagnosis of intestinal protozoal infections—was at least relevant to his main interest, and his pupils became to some extent his collaborators. The Wellcome Bureau of Scientific Research, then in Henrietta Street, London, was the scene of his daily life for the next four years. It was while he was at the Bureau that he was elected a Fellow of the Royal Society (1918); he was still only thirty-two. This distinction pleased him greatly, and he was further delighted when D'Arcy W. Thompson, now one of his heroes, invited him to dinner in celebration of the occasion.

When the war ended and the time approached for him to resume his duties at the Imperial College, he became greatly depressed. He wrote (22 May 1919): 'I can't face another period of routine lecturing and demonstrating—when I ought to be getting on with my own work. I really am unhappy when I contemplate a return to the Academic Life.' So he was overjoyed when the Medical Research Committee (later Council) invited him to accept the post of protistologist at the National Institute for Medical Research, then at Hampstead. Now at last his way was clear. His work on amoebic dysentery during the preceding four years had given him a unique view of the problems—both scientific and medical—that might be solved only by extensive and intensive study, experimental and cytological, of the protozoal fauna of the human intestine. He had a programme of research in mind that would take him years to put through. As things turned out, it occupied him for the remainder of his life. With the warm approval of Sir Walter Fletcher, then Secretary of the Medical Research Council, he embarked on the scheme enthusiastically, in a place where he was on excellent terms with a number of colleagues whom he had come to know and appreciate during his work for the army.

The rest of Dobell's professional life was, on the surface, singularly uneventful. The manner of it, and his seeming unsociability, can only be understood by those who realize that he had deliberately dedicated his whole being to the search for truth within the circumscribed field that he had made his own.

And there must also be taken into account his dislike of present-day methods of attacking biological problems. He abhorred what are called 'research teams', being convinced that, in the sort of work in which he was engaged, they presented occasion for inaccuracy. Moreover, by nature, he was so much of an individualist that he found collaboration extremely difficult except with the chosen few who shared his point of view and would adopt his methods and his standards. He carried this isolationism to the point of refusing to employ the research assistants offered him by the Council; he himself made all his own cultures and examinations, cut all his own sections, stained all his own preparations, and

personally cared for all his experimental animals. The labour this imposed can well be imagined—especially during the years of the Second World War, when London was so vulnerable to bombing that all research within the capital was attended by abnormal difficulties and when the nights were broken by fire-watching duties at scientific institutes. For his own safety he was quite unconcerned, but the frequent failure of gas, electricity and water supplies constantly endangered his cultures and made him frantic with apprehension. There is no doubt that, like so many other civilians of his generation, he was to some extent a war casualty.

Dobell's published work naturally falls into two main periods. There are the papers on general protistology, written prior to his appointment with the Medical Research Council; and then follow the books and papers dealing with the intestinal Protozoa of man. There is only one notable overlap; his completed work on *Aggregata* did not appear until 1925, and in the introduction thereto he pays tribute to the Council's 'enlightened concept' of medical research that had enabled him to finish what he had been engaged on for eleven years before he took over his new duties. To the second period also belong his biographical sketches and his monograph on Leeuwenhoek: but this work he did at home, and generally after midnight.

A glance at his bibliography (pp. 12–15) would suggest that he was interested only in parasites. But while relatively few even of his early papers deal with free-living Protozoa, his knowledge of the phylum (or sub-kingdom, as he preferred to call it) as a whole was quite extraordinary. He published little on the Ciliata, for instance; but actually he knew more about free-living ciliates than do most specialists on the group. Though he gave most of his time to parasites, he considered that knowledge of the structure and behaviour of the free-living stocks from which these presumably have, at some time, been derived, often provides a clue to the solution of problems presented by micro-organisms living in highly specialized environments within the animal body. Moreover, since he was a protistologist, he did not confine his attention to the Protozoa. He was a well-trained bacteriologist also, and was deeply interested both in the cytology of bacteria and in the part they play as biotic factors in the environment of entozoic amoebae. Though he never published anything on the subject, the problems presented by the oscillatorians fascinated him for years.

What strikes one on reading his early papers is the grasp and maturity they display. It is difficult to believe that 'The structure and life history of *Copromonas subtilis*' (1908*a*) and 'Researches on the intestinal Protozoa of frogs and toads' (1909*b*) are the work of a youth of twenty-two. There is already the sureness of aim, the clarity of statement, the lightness of touch, the naturalist's appreciation of his animals, and the recognition of his great predecessors' achievements that marked Dobell's writings throughout his life.

The paper on *Copromonas* gives an account of the whole life history of a new coprozoic monoflagellate common in the faeces of Amphibia, and includes the important discovery of a sexual phase not known before, or since, for any other flagellate of the kind; the diagram that Dobell drew in illustration of this is now in all the text-books. He had found *Copromonas* while engaged in the researches that form the material of a second, more ambitious paper; in that he deals mainly with the flagellates that form so large an element in the amazing microfauna of the amphibian gut but were too small for treatment by the old microscopists who discovered them; and he gives the first worth-while account of *Entamoeba ranarum*, morphologically indistinguishable at all known stages of its life history from the amoeba pathogenic to man, *E. histolytica*—the organism to the study of which he was later to devote so many years. In certain minor matters Dobell's work on the trichomonads has been modified by later observers equipped with new cultural and cytological techniques, and he freely admitted that he had been wrong in attributing an encysted stage to these organisms; but the work is nevertheless a classic of its kind, and it had a healthy influence on a science that had been drifting away from the direct observational methods of protozoologists like Bütschli and Maupas, who saw the micro-organism as a whole and, for most purposes, preferred it living to dead.

During this first period he did service to protozoology in another direction when, in 'Cytological studies on three new species of amoebae' (1914*b*) he demonstrated the futility of attaching specific names to these amorphous organisms except on the basis of their nuclear structure, mitosis and encystment. But without modern culture methods to aid, the labour involved in Dobell's work of thirty-five years ago was so great that it is not surprising if few would-be taxonomists immediately followed up the lead he had given.

We have mentioned but three of the many purely descriptive protozoological papers of his first period. He was also writing on the cytology of the bacteria and on protistological genetics, and was indulging in a series of spirited attacks on the methods of approach to protistological investigation that had so enraged him while in Germany. He showed, for instance, that it is vain to assume that all protists—even bacteria—must reveal a sexual phase if you search for it long enough (1908*d*, 1909*d–f*); and in 'Chromidia and the binuclearity hypotheses' (1909*c*) he ruthlessly exposed the confusion of thought that

at that time prevailed in the Munich school. But unquestionably his most important paper on these lines was his 'Principles of protistology' (1911*c*), to which he himself always attached great importance, saying that it embodied much of his own views on biology and philosophy. He has also left evidence that he intended to expand it considerably at some later date, but, as it proved, he found little time for further polemics of this kind. The 'Principles' aroused great interest and was translated into Russian and Italian. And while some of the arguments and conclusions never met with general acceptance, Dobell's main contention steadily gains credence, i.e. that the Protista stand apart from all other living things, being constructed on a totally different principle: they are acellular, or non-cellular, organisms, and are not, any of them, to be homologized with a single cell of a metazoan or metaphytan body.

Dobell's most important contribution to general protozoology was his monograph on *Aggregata eberthi* (1925*a*). He had been working on this organism for sixteen years before he published his conclusions in their final form (vide supra). As far back as 1908 he had decided that this abundant, digenetic sporozoon, living in cephalopods and in the crabs on which these feed, and so large as to be visible to the naked eye, would repay cytological as well as experimental investigation; and he early came to the conclusion that *Aggregata* was not a gregarine, as Moroff and other contemporary protozoologists maintained, but a coccidium, as Mingazzini, Labbé and, more especially, Siedlecki, in the nineteenth century, indicated. In 1914, foreseeing an interruption to his normal research work, Dobell put out a preliminary note to this effect. The completed record of his work and conclusions, under the title 'The life history and chromosome cycle of *Aggregata eberthi*—(Protozoa : Sporozoa : Coccidia)' occupied 136 pages of *Parasitology* and was accompanied by numerous illustrations, including some 200 of his exquisite water-colour drawings. Within this compass we find not only the full and detailed life history —with, for the first time, a reliable description of the schizogonic phase in the crustacean host—but interesting accounts of experiments undertaken to show how, after its sporogony in the cuttle-fish, the parasite gains access to the crab when the crustacean ingests the oocysts with food contaminated by the mollusc's faeces, and re-infects the mollusc when this eats the crab in which merozoites have been formed. The developmental events in either host form a single, unbroken series. There is no recurrence of the sexual phase in the mollusc until schizogony has taken place, and this asexual phase occurs only once in a given parasite in a given crab. Dobell points out that these circumstances in the life history of *Aggregata* are of inestimable value to the investigator of the Coccidia, and eliminate, from the start, a number of possible causes of error and misinterpretation. He then proceeds to elaborate certain features in the cytology which he thinks may be of general application throughout the telosporidian Sporozoa and which are confirmatory of his opinion that, in nuclear features as well as in their bodily architecture, the Protozoa stand quite apart from multicellular animals. The nucleus of *Aggregata*, he thinks, should be described as a 'nuclear system', for, in his view, every adult is essentially binucleate, with one of its nuclei lying within the karyosome of the other, and he suggests that this is probably true of the Coccidia in general, although hard to demonstrate in the smaller species. Again he raises the question as to the degree to which homologies can hold between the nuclear apparatus of a protozoon and the nucleus of a somatic cell of a metazoon. He further demonstrates conclusively the haploid nature of *Aggregata* at every stage but one of its life history. The chromosomal number is six except in the transient zygotic phase, when twelve are apparent. The first nuclear division in the zygote is meiotic, and thereby the haploid number is re-established. He shows that the six differentiated and very unequal chromosomes of *Aggregata* are recognizable at every nuclear division and that like chromosomes pair before the first division of the zygote: this was no easy task, since, with so large an organism, the longer chromosomes extend over several sections. Dobell found in the chromosomal history of *Aggregata* a challenge to the theory 'that the chromosomes—any or all of them—"determine" morphological characters, "bear" hereditary properties, or constitute "the physical basis of heredity"' in the Protozoa, since male, female and asexual stages of *Aggregata* are all haploid and have apparently identical chromosomal constitution, notwithstanding their morphological and physiological diversity. These facts led him to criticize the accepted views regarding the genetical role of chromosomes in general.

During the years of the First World War, Dobell's publications were mainly concerned with the work he had in hand then, which was the laboratory detection of *Entamoeba histolytica* among the troops. They take the form of contributions to the *Special Reports* issued by the Medical Research Committee between 1917 and 1921. As no epidemiological investigation of amoebic dysentery has since been carried out on a comparable scale, and as little has since been added to our knowledge of the disease caused by this amoeba, these *Reports* still serve as an important source of information for all medical men and parasitologists concerned with this subject. Dobell was closely associated with the investigation of the action of drugs in clearing up the dysenteric condition and in rendering convalescents and

'contact carriers' innocuous. He played a leading part in improving the method of treatment and dosage with emetine bismuth iodide (E.B.I.), which was adopted as a standard by the War Office Dysentery Committee—of which he was a member—and by the Ministry of Pensions.

It was Dobell who elucidated the importance of carriers in the aetiology of amoebiasis, and stressed the significance of harmonious relations between man and the dysentery amoeba for completion of its life cycle in human hosts. He also suggested to his trainees the advisability of examining persons who had never suffered from amoebic dysentery and indeed might never have been out of the United Kingdom. This work, which was subsequently extended to other temperate regions, has shown that probably about 10% of the healthy population throughout the world is infected with *E. histolytica*.

After he became protistologist to the Medical Research Council, his publications—apart from the few we have mentioned above—were exclusively devoted to human parasitology. But, to use his own words—these were always 'the work of a biologist and never of a medical man—of one whose chief interest is, and has ever been, in the Protozoa themselves and not in the diseases they produce'. In his programme, he set himself to investigate man's intestinal Protozoa, and especially the amoebae. By 1914, the causation of amoebic dysentery had been established—notably by the American worker E. L. Walker; and it was on the basis of these American studies that, from 1914 to 1919, Dobell had built up from his own observations a reliable system for recognition of the main protozoal parasites with which the investigator of dysenteric stools might have to deal. It was obviously of great practical importance to use discrimination in diagnosing amoebic dysentery, and since at the beginning of the First World War few were competent to do this, Dobell's first task had been to show how *E. histolytica*—whether free or encysted—may be distinguished from comparable stages of its harmless associate, *E. coli*, and to avoid confusing with these, or with one another, the less familiar Protozoa commonly inhabiting the human intestine. Two more non-pathogenic amoebae were noted—*Dientamoeba fragilis* Jepps & Dobell (1918) and *Iodamoeba bütschlii*; the latter was first accurately described and named by him (1919*b*), and he also put *Endolimax nana* on the map. During the war he had learnt a good deal about the intestinal flagellates of man—especially *Giardia intestinalis* and *Trichomonas hominis*—and had done his best to check the tendency to attribute to them pathogenic properties. His experience had shown him that there was a great need of books to which the investigator of intestinal disorders might confidently refer, and between 1919 and 1921 he published two that are still indispensable guides. The first of these, *The Amoebae Living in Man*, he himself described as 'a zoological monograph', and it embodies all contemporary knowledge of the organisms that his own extensive familiarity with the literature and four years of unbiased first-hand study of human stools could concentrate in small space. Like all his work, it was beautifully illustrated and fully documented; and though thirty years have passed since its publication, this book is still the main guide for the student of amoebiasis. The second book, *The Intestinal Protozoa of Man*, includes also the flagellates, ciliates and coccidia, and deals briefly with coprozoic Protozoa that may be confounded with true parasites. Although this work was published under joint authorship, Dr F. W. O'Connor had little to do with its preparation beyond the initial planning. It, too, is an invaluable handbook, and everyone interested in the subject must regret that it has now been for some years out of print. It contains one blemish that Dobell himself would have been the first to wish removed; in describing the coccidia that may be encountered in the intestine of man, he included three species of *Eimeria*, two of which he himself had noted and named. Subsequent workers have demonstrated that he and others had misinterpreted oocysts of eimeriid coccidia belonging to fishes and merely ingested by the persons in whose faeces they were passed.

And now we come to his work at the National Institute for Medical Research. When he first entered on this, the only method for detection and study of the dysentery amoebae was by microscopical examination of samples of the stools passed by human beings or by artificially infected kittens. While Dobell insisted that at least three specimens of human faeces, passed on different days, should be examined before a verdict of 'negative' was permissible, there was little doubt in his mind that many 'positives' got through the screen. And if this were true of *Entamoeba histolytica*, it applied with greater force to organisms like *E. coli*, the cysts of which tend to be much more unequally distributed throughout the stool. But in 1924 and 1925 Boeck and Drbohlav published the first reliable method for cultivation of intestinal amoebae, and Dobell, seeing the possibilities this opened up, eagerly seized on it and, first alone and then in collaboration with Patrick Laidlaw and with Ann Bishop, so developed and refined it that not only did it become the standard method for detection of slight infections but opened the way to a more profound study of the life histories of the amoebae living in man, and of investigating, *in vitro*, their reactions to experimental conditions. Encystment of the cultural amoebae was induced by the addition of solid rice starch to the medium, and the eradication of the contaminating fungus *Blastocystis* was also made possible. Moreover, the

new media served as bases for testing the lethal effects of emetine on *Entamoeba histolytica*; Dobell and his colleagues were able to show that the drug has a direct effect on the parasite. And after an all-fluid medium had been elaborated wherein the drug was not, as formerly, absorbed by any solid component, the minimum amoebicidal dose of emetine was exactly determined.

Now that it was possible to follow in culture the mode of multiplication of man's intestinal Protozoa, to induce encystment and then hatching at will, and also, by means of emetine, to clear a host of *E. histolytica*, Dobell's way was clearer and he went methodically ahead. Between 1928 and 1943 there appeared in this *Journal* eleven papers under the general title of 'Researches on the intestinal Protozoa of monkeys and man'. In these Dobell dealt with the life histories of certain of the flagellates and with all but one of the five amoebae. Previous to 1928 nothing but the trophic and encysted stages of these had been known. Dobell had long been aware of 'the many serious gaps in our knowledge of these organisms', but was also sure that they 'could be filled in by means of properly planned and carefully executed experiments'. 'A critical survey of the relevant literature', he goes on, 'has already convinced me, moreover, that our present "knowledge" is little more than ignorance and guesswork.... What we want is certain knowledge—however small the amount—derived from accurate observation and critical experiment' (1928).

He was enabled to set about making these observations and experiments only by having first perfected the culture methods we have referred to, by having then discovered precisely how to make sure that he had cleared a suspect host of *E. histolytica* by means of emetine, and lastly by having recourse to study of, and experiment with, the intestinal Protozoa of monkeys, bearing in mind their possible interchangeability with those of the human host. Though exact information was lacking, it had been known for some time that many species of monkeys harbour a protozoal fauna similar to that characteristic of the intestine of human beings; and 'monkeys as a group...are sufficiently akin to us to warrant the assumption...that what is generally true of their protozoal inhabitants is probably true of ours' (loc. cit.). If it could be shown experimentally that the non-pathogenic *E. histolytica*-like organism in monkeys is identical with the organism pathogenic to us, a method of experimentation would be opened up that could not otherwise be put in practice. 'The simian Protozoa are obviously worthy of study for their own sake: and if their possible identity with the corresponding forms in man be considered, they must possess a very real and human interest' (loc. cit.). 'Many people doubtless would be willing to submit themselves to irksome experiment for the sake of advancing knowledge; but probably few would volunteer...for post-mortem examination at the conclusion' (loc. cit.).

Most of Dobell's monkeys belonged to species of the genus *Macacus*, notoriously intractable animals and very difficult to keep healthy in confinement. 'At the outset', he says, 'I realized that it would be impossible to make any satisfactory experiments over a long period with untamed and unhealthy monkeys. I therefore devoted much time to taming my animals and learning how to keep them fit in captivity....I can easily handle and treat them in any way necessary for parasitological investigation, and can obtain their co-operation in any experiment which I desire to perform....They must be kept as pets—not as savage beasts—and each individual's requirements must be studied and satisfied' (loc. cit.).

To readers of this *Journal* Dobell's man-monkey experiments must be so familiar that it may seem unnecessary to say much about them, but the importance of the work is such—on general scientific grounds as well as from the purely practical results they yielded—that we think it well to give some summary of the results. The outcome of his experimentation with *E. histolytica* (1931*a*) was that the simian and human strains are indeed identical, although neither is pathogenic to monkeys; and consequently, that macaques are natural hosts which can be used instead of human beings for study of this parasite. In Dobell's own words (loc. cit.): 'It is now practicable (1) to isolate any desired strain of *E. histolytica* from a man or a macaque, and maintain it *in vitro* for any given period; (2) to obtain at will any stage in its life history *in vitro* at any given moment; (3) to introduce this strain into a natural host (*Macacus*), study its behaviour therein, and recover it in culture whenever necessary; and (4) to eradicate it from this host permanently, if desired, by chemotherapeutic means. Host and parasite can thus be kept separate, can be united, and can again be separated *ad libitum*....I believe that we possess, at present, such complete control over no other animal endoparasite.'

The cultivation *in vitro* of *Entamoeba histolytica* led not only to development of methods that would induce its encystment at will, but also to the complete elucidation of what Dobell called its 'metacystic' development—hitherto unknown. It had been a mere guess that the quadrinucleate cyst, when received into the human gut, hatches and gives rise to four amoebulae. Dobell was the first to observe the excystation *in vitro* and the emergence of a single quadrinucleate amoeba which, by a series of complicated nuclear and cytoplasmic divisions, ultimately and surprisingly gives rise to eight uninucleate amoebulae. No one who has not attempted the task of tracing the fate of the hatched-

out contents of an amoebic cyst can have any idea of the patience and the technical skill required in order to get the complete story clear. When Dobell's experiments did not give the anticipated results he always faced his disappointment philosophically, and used to say: 'It's only when things happen differently from what we expect that we begin to learn.'

While the work on the pathogenic amoeba could readily be appreciated by his medical colleagues, Dobell found it less easy to interest them in the remainder of his programme; but, as a man of science, he considered it important that the whole biotic environment of *E. histolytica* should be fully investigated, and he was not content until he had subjected the other amoebae of the human and macaque intestine to similar culture and experimentation. In some ways his work on *E. coli* (1936*c*, 1938*a*), the commensal so frequently occurring alongside *E. histolytica* in both man and macaques, seems, to the protozoologist, even more remarkable than that on the pathogenic organism. The difficulties in cultivating *E. coli* proved almost insurmountable. After 1925, it took Dobell twelve more years before he was satisfied that, here also, he had the whole story. He refused to be beaten—quoting Kipling's 'as soon as you can do anything, do something you can't'. Needless to say, he finally scotched the Schaudinn-inspired story of an autogamous phase, and he showed that in the metacystic development the octonucleate amoeba emerging from the cyst behaves very differently from the excysted *E. histolytica*. Moreover, he made it clear that, *in vitro* at any rate, some strains of *E. coli* actively ingest red blood corpuscles—a habit previously regarded as 'diagnostic' of the pathogenic amoeba alone. This observation, as Dobell himself mildly remarks, 'is one of very great practical importance'. Since *E. coli*, from whatever primate source—man, macaques and marmoset—is, however, non-pathogenic, the possibility of establishing specific identity by cross-infection made the experiments in this case absolutely conclusive; Dobell himself acted as the human recipient.

He did this again in the case of *Endolimax nana* of macaques (1933, 1943). Having grown this amoeba in pure culture and established that he was uninfected, he inoculated himself with the cysts from a macaque and, at the time of writing the 1933 paper, had maintained the induced infection unchanged for eight years. From himself, he transferred this *E. nana* strain to an uninfected macaque, where it persisted for the rest of the animal's life, 2½ years.

Dientamoeba fragilis from man and macaques astonishingly proved, in culture, to be an aberrant flagellate, closely allied to *Histomonas*—the causal organism of Blackhead in turkeys. It never encysted, and his attempts to infect man and monkeys, by inoculation with the trophic amoebic stage were unsuccessful. Dobell suggested (1940*b*) that, on analogy with *Histomonas*, the mode of transmission of *Dientamoeba* might eventually prove to be through the ova of a nematode worm.

The only remaining amoeba of the intestine of man and monkeys is *Iodamoeba bütschlii*. It is clear, from his letters to us and from the *Report of the Medical Research Council* for 1939–45, that he had been working on it for a long time and was at last making good headway, having after many attempts been able to cultivate it continuously. In 1947 he had studied all its division stages but was having trouble with the encystment. However, in that too he eventually succeeded, and just a few months before his death he told us that he had also completed a study of the excystation and metacystic development; the results would surprise us very much, he remarked. But after his death, no trace of a manuscript or drawings dealing with the life history of *Iodamoeba* could be found among his orderly papers: the slides were there and his daily notes relating to the work—and that was all. We can only conclude that he had been following his usual custom in his professional writing, of eschewing rough drafts until the very end of the work when, relying on his amazing memory for detail, he would at last commit the whole to paper.

In these last years he was also putting to fuller use his extensive bacteriological knowledge, with special reference to the part bacteria play in the environment of intestinal amoebae. Quite early in the course of his work for the Medical Research Council, he turned his attention to the influence of the bacterial flora on his cultures, and showed (1926–31) that some species are favourable and others harmful to successful growth *in vitro*. Indeed, certain bacteria seemed to have a determining effect upon the life history of the dysentery amoeba, and he was eventually able to manipulate the flora in such a way as to induce cyst-formation by this means. Latterly he was becoming so expert in this line of work that, in the *Report of the Medical Research Council* for 1945–8, there appears the important statement that 'He has now found it possible to grow the amoebae [*Entamoeba histolytica*] in pure mixed culture with one or more known species of bacteria (instead of in the usual way with a complex and unknown mixture of bacteria), and so to ascertain the part played by each species and its effects on the development of the amoebae. Many such pure strains, of known and accurately determined bacterial composition, have now been made and studied in detail. An outcome of this work has been the preparation of pure cultures of the amoebae themselves—unaccompanied by any other living organisms—and the methods of obtaining and propagating these are being further investigated.' Fortunately this work will not suffer

the fate of the researches on *Iodamoeba*; Dobell had finished it, and he was making a record of it at the time of his death. This record, which was to be the twelfth instalment, in this *Journal*, of his 'Researches on the intestinal Protozoa of monkeys and man', though incomplete had reached a manuscript stage from which, with the help of the full table of contents that he had set out and the daily records extending over many years, the missing parts have been reconstructed, and it has been possible to publish (1952) the last contribution to the subject to which this man had literally given his life.

It remains to give some account of Dobell's publications on the history of his science. Here we have had the generous assistance of Prof. F. J. Cole, F.R.S., on whose notes we have drawn freely in what follows. Very early, when he was first studying the Protozoa of Amphibia, Dobell became deeply interested in past records, and was amazed to find that these went right back to work by Leeuwenhoek in 1683 and 1702. He began to study Leeuwenhoek systematically and discovered that the old Dutch draper had described some thirty-five species of Protozoa so well that most of them could be recognized by the twentieth-century specialist. He (1915) pointed out that in 1683 Leeuwenhoek had described a case of Mendelian dominance in the rabbit, and he (1920) showed that indubitably it was Leeuwenhoek who first observed intestinal Protozoa in man; the descriptions made it possible to identify one organism as *Giardia intestinalis* and another as *Chilomastix* or *Trichomonas*. He found (1920) that, as early as 1674, the oocysts of the coccidium *Eimeria stiedae* had been recorded by this remarkable man, whom he henceforth regarded as 'the father of protozoology'. Dobell's inherent love of old books was so stimulated that he became an ardent collector and, in the course of years, his knowledge of what was worth while in booksellers' catalogues enabled him to build up a very fine collection at comparatively small expense. His election to the Royal Society gave him access to valuable material in its Archives, and he found there many of Leeuwenhoek's early letters, among which he announced (1931*b*) the discovery of thirty-two that had been supposed lost and so had not been included in the Dutch and Latin collected editions of Leeuwenhoek's works. All this material is incorporated in the complete correspondence now being published in Holland by the Leeuwenhoek Committee.

Dobell became obsessed with Leeuwenhoek, and for years devoted most of his spare time to preparing his great monograph *Antony van Leeuwenhoek and his 'Little Animals'*, which was published in 1932 and is generally regarded as a masterpiece in its *genre*. His 'spare time' in which this was accomplished was 'mostly at the dead of night...after a hard day's work in the laboratory at my own research, and with another similar day in the laboratory before me'. His remarkable powers of application and the untiring devotion he brought to any piece of work on the completion of which he had set his mind, do not alone account for his ultimate success. He had great historical insight and literary and linguistic gifts of high order. On these powers a study of Leeuwenhoek made exceptional demands, for the old naturalist writes in the obsolete Dutch of the seventeenth century, while his style is eminently personal and colloquial; he employs homely expressions and similes, not to be found in any dictionary. In remarkable fashion, Dobell identified himself with the dead man and his aims, and, with his own vast knowledge of protistology to aid, produced, in time for the Leeuwenhoek tercentenary in 1932, the fascinating, original and vital work that has since taken its place among the famous classics of its kind. Leeuwenhoek's countrymen have been among the most enthusiastic appreciators of this great tribute. To mark the tercentenary celebrations a bronze medal was struck, of which Dobell was sent a silver replica. Dr W. H. van Seters (1950), in his *In Memoriam* notice of Dobell writes of the part he played in making Holland again Leeuwenhoek-conscious: 'We should not forget that it was Dobell, an Englishman, who prepared this "renaissance" and made it possible.' While preparing the monograph, Dobell made a unique collection of notes, cuttings, pamphlets, etc., pertaining to the subject; and these, the *Leeuwenhoekiana*, have, in accordance with his expressed wish, been presented by his widow to the Royal Society.

While his gift as a biographer was mainly dedicated to Leeuwenhoek, Dobell wrote many shorter appreciations of biologists whose names he thought deserving of tribute or commemoration. Most of these articles appeared either in this *Journal* or in *Nature*: among them are those on T. R. Lewis, L. Joblot, C. G. Ehrenberg, B. Grassi, R. v. Hertwig, V. Lambl, M. Siedlecki, F. Vejdovsky, H. Drbohlav, H. S. Jennings, C. S. Kofoid, W. Bulloch and Bütschli. They are all marked, not only by careful documentation and precision of report, but by what Dobell's detractors would have most denied him—the power to enter into another man's point of view. One of the last he ever wrote was the best of all when, in 1949 and just before his own death, he paid a masterly tribute, in an obituary notice for the Royal Society, to Sir D'Arcy W. Thompson; the close friendship between these two very unlike men had extended over thirty years, and Dobell, grief-stricken by the loss of one he venerated so profoundly, reveals himself in his most human aspect.

Under the guise of a biographer, he could be slightly malicious, however, as in his satirical

'obituary' notice of 'Dr O. Uplavici (1887–1939)', in which he revealed the perpetuation, as the name of an author, of the Czech title of a paper 'O úplavici' (=on dysentery). And he enjoyed exposing—as in '*Teranympha* and other monstrous Latin parasites' (1939*b*)—the absurdities into which ignorance of dead languages may lead the scientific man. He also did a very ingenious bit of detective work when he discovered and then traced (1947*a*) the history of a manuscript volume entitled *Kincardine papers: Letters from Robert Murray to Alexander Bruce*. This valuable correspondence of Sir Robert Moray, the first President of the Royal Society, Dobell presented to its library.

Although he professed to dislike attending 'meetings', saying that they were 'waste of time', and as a result was far too little known personally to his fellow-workers, it was inevitable that Dobell should be a member of many scientific societies, and he especially appreciated the honorary foreign Fellowships—such as that of the R. Accademia dei Lincei of Rome—that were conferred on him in the course of time. He served on a number of committees—four of them in the Royal Society of London, and he was a member of the editorial panel of *Parasitology*. We are told by Prof. D. Keilin, F.R.S., the editor-in-chief, that, as might have been expected, Dobell did not like editorial work—'simply because no paper he read could satisfy him. He had always an urge to re-write it completely, a task for which he could find no time.... He was always very helpful and generous to the author, offering him valuable information and never expecting any acknowledgement of it.'

His own contributions to this *Journal* began in 1908, and the last appeared in 1943. After 1917 there was something from Dobell in almost every volume, and when the fine series of 'Researches on the intestinal Protozoa of monkeys and man' started in 1928, they came to form a 'feature' to which all protozoologists looked forward. Though the layout of the typescript and the execution of the illustrations were faultless, he cannot have been an easy contributor to deal with, for he would brook no interference with his methods of presentation, or any elimination of the carefully chosen literary quotations with which his papers were headed or of the footnotes that, as an American colleague (Dr Gordon H. Ball, 1950) remarks in his obituary, 'bring an added zest to an already vital page'. In the years of acute paper shortage it must have been very difficult to make the editorial adjustments that these contributions by Dobell demanded, but it was worth doing since, however lengthy and detailed, there was real economy at the back of the whole clear-headed plan, and future workers on the entozoic amoebae should have their way made much easier for the pains he took to leave nothing in his methods obscure.

In the course of his published writing, his caustic criticism of the work of others earned him many enemies, and his seeming arrogance was bitterly resented. But, again, to quote Dr Ball: 'His very honesty and sincerity militated against the use of "diplomatic" language or the glossing-over of what he considered to be shoddy scientific work.... Actually he was more generous with his praise for work well done than he was with his criticism of what he believed had been poorly done. His first love was science.' He was always lavish with his help to those who appealed to him. Only a very generous man would have given so much of his precious 'spare time', between midnight and 3 a.m., to answering—and usually by return of post—the innumerable letters he received from all over the world asking for help and advice on this or that. No doubt this correspondence partly served to fill some need for the human contacts that his day's work at the Institute made less and less possible.

His was a highly emotional nature; he has said of himself that he was cursed with the artistic temperament 'with all its excesses and defects' (1949) and the regime he imposed for faithful execution of his purpose as a man of science might have been more harmful but for some inconsistency in its absolute application. Very conscious of the demands made by human relations and their interference with the main object of his life, he strove for years to steer clear of all commitments other than those arising from filial duty. The necessary condition for really good work, he considered, was complete immunity from all entanglements with other people's lives; and although always a fatalist, even to the point of superstition in minor matters, he endured much mental distress when important decisions had to be taken. Nevertheless—rather to his own surprise, perhaps—he accepted, in 1937, the risks and responsibilities of marriage. His wife was Monica Baker, step-daughter of his close friend, the late Prof. William Bulloch, F.R.S. This venture proved very happy, and in his home Dobell, for the last twelve years before his death, had the devoted support and companionship of one who, while not herself a scientist, appreciated his aims and actually promoted the freedom he longed for.

This warm human relationship was the more necessary for him since, as he saw the end approaching of his tenure of the post as protistologist to the Medical Research Council, he became increasingly anxious to finish his task there in good style, and put more and more pressure on himself to achieve the seemingly impossible before he reached the fateful age of retirement. Thoughts of a future unconnected with any possibility of laboratory work preyed greatly on his mind, as his letters to friends from 1945 onwards show. He could not conceive of a satisfying life away from his microscope, and those of us who

had his happiness at heart must be thankful that he was spared the dreaded divorce from his chosen occupation. When he suddenly died, he was still at the height of his mental powers, and had much to do and to give, but it cannot be doubted that his published works will remain an enduring monument to the greatest protozoologist of his time: in his works he shall live.

Acknowledgements. Among the sources of information upon which we have drawn for Clifford Dobell's biography are the reminiscences kindly placed at our disposal by a number of his friends and admirers, whose help we have gratefully acknowledged elsewhere (Hoare & Mackinnon, 1950). We now desire to express our appreciation of further help, received from persons named in the present article.

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RESEARCHES ON THE INTESTINAL PROTOZOA OF MONKEYS AND MAN

XII. BACTERIAL FACTORS INFLUENCING THE LIFE HISTORY OF *ENTAMOEBA HISTOLYTICA* IN CULTURES

By the late CLIFFORD DOBELL, F.R.S., *National Institute for Medical Research, London*

Completed by R. A. NEAL, Ph.D.

Edited by CECIL A. HOARE, F.R.S., *Wellcome Laboratories of Tropical Medicine, London*

(With 1 Figure in the Text)

Ad opera nil aliud potest homo, quam ut corpora naturalia
admoveat et amoveat: reliqua Natura intus transigit.
Bacon, *Nov. Org.* I, iv.

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Foreword

When he started his researches on *Entamoeba histolytica*, the late Dr Clifford Dobell, F.R.S., soon discovered that bacteria had an important influence upon the growth of the amoebae in culture, which was promoted by some species and inhibited by others. He also showed that certain species have a determining effect upon the life history of *E. histolytica* and by manipulating the bacterial flora he was able to induce cyst-formation. He eventually completed his investigations on this problem, and in the *Report of the Medical Research Council* for 1945–8, he announced that 'He has now found it possible to grow the amoebae in pure mixed culture with one or more known species of bacteria (instead of in the usual way with a complex and unknown mixture of bacteria), and to ascertain the part played by each species and its effects on the development of the amoebae. Many such pure strains, of known and accurately determined bacterial composition, have now been made and studied in detail.'

During the last years of his life—up to the time of his sudden death on 23 December 1949—he was engaged in making a record of this work. After his death, a draft manuscript was found bearing the title of the present paper. Though provided with a full table of contents, and even accompanied by one of his aptly chosen mottoes, this manuscript was unfortunately not completed. However, with the index as a guide and with the help of the daily records which Dobell had kept for many years, Dr R. A. Neal succeeded in reconstructing the most essential parts of the missing portion, and we were thus able to prepare for publication Dobell's last contribution to a subject to which he devoted the best years of his life.

The first and greater part of this paper (pp. 16–34) contains Dobell's original account of his own work, while in the second part his observations are described by R. A. Neal.

C. A. HOARE

I. INTRODUCTION

This is a preliminary study of a subject both vast and complex, and it offers no final solution of any problem. It is the outcome of work upon which I have been engaged for the last 20 years in conjunction with other studies already partly published in

earlier instalments of these *Researches*. But it is not a 'preliminary communication' in the sense that it is premature: rather is it a 'progress report' of work unfinished but still in hand—work done, undone, and redone repeatedly over a long period and in circumstances which have seldom been favourable. In its final stages, indeed, the vicissitudes of war have sometimes been almost fatal to its fulfilment.

Most of the observations and experiments which I am now about to describe were made—but not published—more than 12 years ago. I have since checked and repeated them many times, but have never been able to 'complete' them to my own satisfaction. But in the meantime others have studied the same subject, though from a different standpoint, and have published their results. As these differ in many ways from mine, and seem to me to overlook many important points I have resolved to put my own findings on record. So they are admittedly incomplete and faulty; but the subject is one without an end, and will not be finally elucidated in my lifetime.

The foundations of this work were laid in four earlier papers (Dobell & Laidlaw, 1926; Dobell, 1927, 1928, 1931)—to all of which, I think, too little attention has been paid by later investigators. The facts recorded in these publications are, at all events, fundamentally important for all students of this subject. I therefore find it necessary to direct the reader's attention to their existence. I can hardly be expected to admire or approve—or even to acknowledge—many newer 'discoveries' already recorded, anticipated, or disproved in my own earlier writings.

I began this work with the late Sir Patrick Laidlaw, F.R.S., and I owe much to him for his collaboration in early days. From 1924 until about 1927 we were most closely associated, and he continued to give me help and advice for several years afterwards. Later, he lost interest in our originally joint work—through preoccupation with other studies of his own—but to the end of his life (he died suddenly on the night of 19–20 March 1940) he lent me aid and apparatus and reagents which were invaluable. My debt to him is therefore acknowledged gratefully. I would also record my indebtedness to another former colleague—the late Capt. S. R. Douglas, F.R.S., who gave me help and encouragement till the day of his death (20 January 1936). To both these friends I have been privileged to demonstrate my findings on innumerable occasions.

As an instance of the neglect or misunderstanding from which my early work with Laidlaw has suffered, I may perhaps be allowed to quote the following. In our paper of 1926, we recorded that *Entamoeba histolytica* could be cultivated under 'strictly anaerobic' conditions. Later, Cleveland (1928) claimed to have cultivated a trichomonad similarly, and said (p. 275): 'Incidentally, it should be mentioned that, as far as I know, this is the first instance of a protozoon being able to live and multiply under strict anaerobic conditions.' But the charge is unfounded. Laidlaw had had great experience in the cultivation of anaerobic bacteria before we published our paper in 1926, and was the inventor of the principle incorporated in the 'anaerobic jar' of Fildes & McIntosh. I think we had both studied the cultivation of anaerobes while Cleveland was still at school.

As some of my earlier work was done in collaboration with Laidlaw—and is even published in our joint papers—I have thought it best to signalize this whenever it appears desirable. The reader will therefore please note that, in the following pages, I sometimes write 'we' and sometimes 'I', in recording results or experiments. Whenever I use the first person plural, the findings are those of Laidlaw and myself together; when I use the first person singular, they are my own (though often seen and approved by him). I adopt this method not to claim 'priority' for 'discoveries', but in order to avoid needless repetition and cross-references.

I speak from experience—and greater experience, probably, than that of any other worker; for I have cultivated and studied *E. histolytica* almost every day for 20 years, and must by now have handled more amoebae of this species than any other investigator. If my knowledge is defective, it is not for lack of experience. In the course of my work I have isolated and studied many hundreds of strains. Some have been kept for a few days only, some for several weeks or months, and some for years (up to 18).

II. EXPERIMENTAL

I shall now give a brief account of my experiments and results. These are presented in a more or less logical sequence—which is not the order in which they were done; for many lines of inquiry have been pursued simultaneously over a period of years, and many led nowhere. Results obtained later often qualified or contradicted those obtained earlier, so it is unnecessary to confuse the issue by recording all my mistakes.

Before I describe my experimental findings, however, it seems desirable to make a few general remarks. These, if studied carefully, will help the reader to understand what follows.

(1) General considerations

(i) *The Boeck tube*

A word must first be said about the peculiar type of culture used in most of the experiments described. It is, in principle, different from anything commonly

employed in bacteriological studies, and has curious properties which are not always properly appreciated.

A culture-tube of the type to be considered (Fig. 1) consists of two parts—solid (S) and liquid (L). The solid is in the form of an ordinary

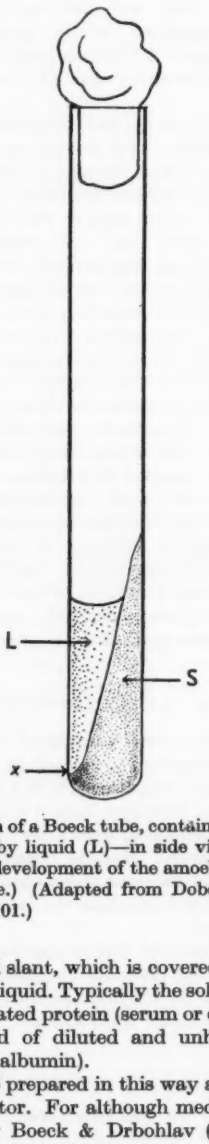

Fig. 1. Diagram of a Boeck tube, containing a solid slope (S), covered by liquid (L)—in side view; x indicates point where development of the amoebae takes place. ($\frac{1}{3}$ Actual size.) (Adapted from Dobell, 1942; *Parasitology*, **39**, 101.)

bacteriological slant, which is covered to a variable extent by the liquid. Typically the solid is composed of heat-coagulated protein (serum or emulsified egg) and the liquid of diluted and unheated protein (serum or egg-albumin).

I call a tube prepared in this way a 'Boeck tube' after its inventor. For although media of this sort were used by Boeck & Drbohlav (1925) for the cultivation of *E. histolytica*—and such cultures are therefore appropriately called 'Boeck-Drbohlav cultures' (to distinguish them from those in liquid media)—they were devised originally to cultivate *Enteromonas* and other intestinal flagellates, by the American worker alone (Boeck, 1924).

To cultivate *Entamoeba histolytica* in such a tube, a small amount of intestinal contents, or other material containing amoebae, is inoculated with a capillary pipette into the liquid at the bottom; and here alone, at the junction of the solid, liquid and glass (Fig. 1, x) development takes place.

Cultures of *E. histolytica* so made are usually incubated at 37° C. 'aerobically'; that is to say, they are exposed to the air, and not protected in any way from free access of oxygen. Nevertheless they are—as will soon be evident—*anaerobic* cultures (so far as the amoebae are concerned). This was obvious to us almost from the start, and we soon found—as anticipated—that the amoebae would develop equally well in such cultures under strictly anaerobic conditions—contrary to the experience of Boeck & Drbohlav.* The aerobic intestinal bacteria which inevitably accompany *E. histolytica* in ordinary Boeck-Drbohlav cultures form a reducing system which promotes the growth not only of the amoebae but also of numerous anaerobic bacteria. This is easily demonstrated. Every flourishing culture of *E. histolytica* is found to contain—even on rough analysis—a complex mixture of aerobic and anaerobic bacteria, while a drop of a sterile solution of methylene blue added to any such culture is rapidly bleached.

Moreover, the Boeck tube is effectively buffered by its solid component. We pointed this out long ago (1926), but the fact seems often to have been overlooked. Any acid or alkali produced by concomitant bacteria is rapidly neutralized—within wide limits—by the Boeck system itself, so that other adjustments are unnecessary.

The original Boeck tube, therefore, affords a means of cultivating *E. histolytica* anaerobically—under seemingly aerobic conditions—and supplies a buffering and reducing system by its very constitution. And it is thus clear that many of the modifications which have since been advocated are merely mistakes—such as buffering with phosphates (unnecessary); addition of alkali (to counteract acid-production by bacteria), or of dextrose (which is only split by the bacteria to form more acid), or of reducing substances (unnecessary); or—strangest of all—coating with liquid paraffin (to produce 'anaerobiosis'). All these and many other 'improvements' appear to be based on obvious misconceptions of the properties of the Boeck tube.

The only real improvement in the Boeck system, since its introduction, consists in the addition of

* See Dobell & Laidlaw (1926), etc. I note this here because certain workers in America have questioned our statement and claimed the 'discovery'. When we wrote 'strictly anaerobic conditions' we meant what we said.

solid rice-starch to the liquid (after the eradication of starch-splitting bacteria). We originated this in 1924, and published our findings 2 years later.* That the improvement is real is testified by the fact that other workers have since claimed the 'discovery', while rice-starch is nowadays an essential ingredient of every 'new and improved' medium proposed. Solid starch supplies a necessary source of carbohydrate—available to the amoebae alone—in place of the original sugar in the medium (dextrose, which is rapidly split by accompanying bacteria).

How far the conditions developing in a Boeck tube during incubation reproduce those in the interior of the living intestine is a matter for conjecture. All that can be said with certainty at present is that the tube provides nutriment suitable for the continued existence simultaneously of a multitude of intestinal micro-organisms—with some control of hydrogen-ion concentration and oxidation-reduction potential sufficiently like the conditions prevailing in the bowel to enable many of these organisms to undergo their complete development outside the living body. Though all the properties of the tube may not yet be known or appreciated, this much at least has been demonstrated.

Note on culture media. Although the various media of Boeck-Drbohlav type which I commonly employ have been repeatedly described already,† I give here (Table 1) a brief synopsis of them—with the symbols used to denote them—for the convenience of readers unacquainted with my earlier papers.

It seems desirable to note also that none of my media is sterilized in the autoclave, and only *fresh* eggs and fresh *whole* (undried) horse-serum are used in their preparation. Media compounded from reconstituted serum, heated above 80° C., and sterilized in the autoclave, are essentially different from those advocated by us (Dobell & Laidlaw), and should not be associated with our names.

(ii) *The constitution of the Boeck-Drbohlav cultures*

It will be evident that ordinary cultures of *E. histolytica* in Boeck-Drbohlav media consist not only of amoebae and culture-medium (solid and liquid) but also of a concomitant bacterial flora. They are—or should be—'pure' *strains* of amoebae, but are 'impure' *cultures* (in the sense that they do not consist of only one species of micro-organism). Nevertheless, properly prepared and cultivated strains of *E. histolytica* are not inconstant and variable mixtures. They are constant combinations of organisms—growing in equilibrium—whose behaviour is predictable and invariable under constant conditions.

The last words are important. No strain of amoebae can be regarded as constant and true to type unless it is kept—after establishment as an entity—under constant conditions of temperature, in standardized and properly sterilized media, and guarded against contamination by extraneous micro-organisms. Although every strain contains an unknown mixture of bacteria, it must be cultivated as a 'pure' culture. Only so will its properties remain invariable.

Freshly isolated strains usually grow irregularly—even under the most carefully controlled conditions—for a variable number of cultural generations. They must frequently be 'doctored' and 'nursed' to rid them of starch-splitting bacteria and other undesirable constituents (and, of course, of other species of amoebae and other protozoa and *Blastocystis*), but sooner or later they 'settle down' and grow consistently, and thereafter they can be propagated *ad libitum*—provided that *the conditions are kept constant* and *contamination is rigorously avoided.*

Table 1. *Culture media*

Designation	Composition: Solid	Composition: Liquid
Ehs	Whole egg emulsified in Ringer's fluid and set by heat (80° C.)	Inactivated horse-serum diluted with Ringer's fluid (1 : 8)
Ere	Ditto	Egg-white diluted with Ringer's fluid
HSre	Whole horse-serum inspissated by heating (80° C.)	Ditto
HShs	Ditto	Inactivated horse-serum in Ringer's fluid (1 : 8)
hs	None	Ditto

When solid rice-starch is added, the symbol denoting the medium is followed by '+S'. Thus 'HSre+S' means horse-serum (solid) covered by Ringer-egg-white (liquid) to which solid starch has been added. (As will be seen, capital letters are used for solids, small letters for liquids.)

* Dobell & Laidlaw (1926). I was amused to read (*Obituary Notices of Fellows of the Royal Society*, 1941, 3, p. 440) that our improvement was 'very characteristic...of Laidlaw's biochemical instinct and ingenuity', for the idea was not his—as he would have been the first to acknowledge—though we worked it out together and published our results jointly.

† See especially Dobell & Laidlaw (1926).

It is important to realize that all strains of *E. histolytica* at present cultivated by the Boeck-Drbohlav technique *in vitro* are different. The amoebae in all are of the same species, and the culture-media employed may be identical, but the accompanying bacterial flora can never be the same in any two strains. The methods of isolation and cultivation automatically insure this result. The bacterial flora, though it is (or should be) constant in every pure strain of amoebae, is *different in every strain*, and this forms an unknown factor of great importance when one strain of amoebae is compared with another.

For this reason it has been my practice to distinguish all my cultivated strains of *E. histolytica* by characteristic letters and numbers. This has been criticized, so I may now answer the criticism. The situation is briefly this: when I grow a strain *X* of *E. histolytica* and with it infect an animal (*A*) experimentally, I call the recovered strain of the same amoeba *XA*. A friendly critic in U.S.A. says that I am mistaken. When *he* passes a species of Bacteria (*Y*) through an animal, and recovers it in culture, it is still strain *Y*, and should be so called. It may have been modified, but it is still *Y*—not *YA*. But this argument is based on a misconception of the constitution of cultures of *E. histolytica*. The bacterial *cultures* of species *Y* are *pure*, but those of my amoebae are not. No two are alike; they all differ in their concomitant bacterial flora, so that *a pure strain of amoebae* can never be identical with its derivative—equally pure amoebically—which has passed through an animal and been re-isolated with a totally different flora. The newly isolated strain is *a new strain*, with new properties, and therefore requires a differential designation.

(iii) *The role of the bacterial flora*

As we pointed out long ago, the bacterial flora, in any culture of *E. histolytica*, exercises a predominating influence on its development and welfare. Our preliminary studies showed that some species of bacteria are favourable for the growth of the amoebae, while some are harmful, and I found out later that certain species may even have a determining influence upon the life history of the amoebae *in vitro*. This observation, indeed, was the starting-point of the present investigation, so I must recall the facts at this point.

Twenty years ago it was a triumph to cultivate *E. histolytica* at all, and the earliest workers were satisfied with their limited success. It soon became clear, however, that it was not only possible to maintain this species alive and growing in its amoeboid form for an indefinite period—as Boeck & Drbohlav had shown—but even to make it pass through all the stages of its life history *in vitro*. We found that by adding starch to the cultures we could —sometimes, at any rate—cause the amoebae to encyst; I then found that the cysts so formed could live for a considerable time at low temperatures and be hatched—and the resultant amoebae be cultivated further—at will.

Further study showed that it is not possible to do this with every strain of *E. histolytica*. The first with which I succeeded completely (strain *K* 28*c*) was isolated in 1926, and formed the basis of my account of the whole life history of this species.* But many other strains—isolated and cultivated in an identical way—refused to form cysts regularly *in vitro*. Usually they encysted less readily, produced smaller crops of cysts, and often ceased later to form any cysts at all. Many strains, moreover, proved to be non-encysting strains from the beginning. The amoebae would grow abundantly, *ad infinitum*, but they would not complete their life cycle in the culture tube.

What are the factors which determine whether a strain of *E. histolytica* will or will not complete its life cycle *in vitro*? Since all strains can be cultivated in the same way, and in the same media, it seemed reasonable to seek the difference between encysting and non-encysting strains in the known variable factor—the concomitant bacterial flora. It was easy to test this hypothesis experimentally, and on doing so I found at once that all my non-encysting (or poorly encysting) strains could be converted immediately into good encysting strains by the simple expedient of growing them with the flora accompanying strain *K* 28*c*. Thereafter they acquired all its properties.

I used this method extensively in order to obtain cysts for experimental purposes, and to study the life history of the amoebae. But is was obviously 'unscientific'—though extremely practical and useful—because I was dealing always with a constant which was really an unknown. The flora in *K* 28*c* itself, and in every other strain, was a complex mixture—an 'impure' culture. It had constant and predictable properties in certain circumstances, but was otherwise indefinable.

I may note here that the action of the bacterial flora in strain *K* 28*c* appears to be specific for *E. histolytica*. When added to cultures of other species of amoebae (or other Protozoa) from Man, it does not cause them to encyst, or determine the course of development *in vitro* in any way. I have verified this by numerous experiments made with *Entamoeba coli*, *E. gingivalis*, *Endolimax nana*, *Iodamoeba bütschlii*, *Dientamoeba fragilis*, *Trichomonas hominis*, *Chilomastix mesnili*, *Embadomonas intestinalis*, *Enteromonas hominis* and *Balantidium coli*. The bacterial mixture is favourable for the growth of all these species—except, perhaps, *Enta-*

* See Dobell (1928).

moeba gingivalis—but does not induce encystation in any of them.

It is hardly possible at present to study the role of the bacteria, in any cultivated strain of amoebae, in every detail. The oecology of such very complex mixtures can be only roughly determined. We know already, however, that some of the concomitant bacteria serve as food for the amoebae—since they ingest them readily; others have a favourable or unfavourable action by altering the reaction of the medium—by producing acid or alkali as products of their metabolism; others establish and maintain physical or chemical conditions necessary for the existence of the amoebae—for example, by removing oxygen, and so promoting anaerobic development. A detailed consideration of all these complex interactions is beyond the scope of the present inquiry.

The main object of the present researches has been to analyse the bacterial flora of strain *K* 28*c*, and to ascertain what part each constituent plays in the life of the amoebae. The investigation is difficult, as most of the problems involved cannot be attacked directly—for reasons which are fairly obvious. New methods had to be devised, or old ones modified, so much of my earlier work proved fruitless, or has been superseded by later and more accurate techniques. I shall not trouble the reader, therefore, with records of my first failures and mistakes.

(iv) *The bacterial flora of strain K* 28*c*

Strain *K* 28*c* was selected for special study for several reasons, though I have devoted much attention to other strains also. First, the behaviour of the amoebae in this mixture was known with considerable certainty. Secondly, the effect of the flora as a whole, when added to other strains, was also known. Thirdly, the flora of this strain seemed—after a preliminary comparative study—to be less complex than that of many others, and therefore likely to prove less troublesome to analyse.

As regards the origin of the bacterial flora accompanying strain *K* 28*c*, I need note only the following data.* This strain of *E. histolytica* was isolated from the dysenteric stool of a kitten (No. 28) experimentally infected (12. ix. 26) with the amoebae naturally living in a macaque (*Macacus sinicus* [=*radiatus*] ♂—my tame monkey 'Bonar'). Cysts in this monkey's normal faeces were washed and concentrated in water; treated with 0·2 % HCl for 1½ hr. at room-temperature; and then fed to the kitten, which contracted dysentery 6 days later. Strain *K* 28*c* was isolated in HSre + S medium on 18. ix. 26. It was found to be pure from the start and was never treated with acriflavine or other antiseptics (to remove starch-splitting bacteria). After hatching the cysts first formed, the strain was kept in Ehs medium. I have kept it most carefully ever since its isolation, and it has never left my hands. It has never been entrusted to the care of assistants or colleagues, and has been most scrupulously guarded against contamination. Very many lines have been propagated during the last 18 years, and all which were even suspected of contamination have been discarded immediately.

It appears certain, therefore, that the amoebae of this strain are of simian origin, but that its bacterial flora consists of a mixture of species derived from the intestine of a kitten; yet the possibility that some of these are ultimately of simian origin also—having been introduced into the kitten accidentally with the amoebae—should not be overlooked. Owing to the method of infection adopted, however, this possibility appears remote.

I have already noted that the properties of the flora in strain *K* 28*c* were known. By this I mean that this strain of *E. histolytica* behaved (and still behaves) in a regular and predictable manner when grown under constant conditions with its naturally present bacterial admixture, and that these accompanying bacteria are capable of conferring the same properties on other strains when added to them. The methods adopted for obtaining and cultivating the flora *en masse* (regardless of its composition) and for studying its effects on other strains, may conveniently be noted here.

The bacterial flora as a whole can be easily cultivated separately—free from amoebae—in various ways. Originally I obtained it, as a separate and stable mixture, in the course of numerous attempts to test the viability of amoebae and cysts under various conditions.* By keeping cultures of amoebae at low temperatures, for example, I determined their time of survival; after this, cultures and subcultures were, of course, 'negative' (for amoebae). But they contained all the bacteria accompanying them, and these could then be propagated *en masse* in the same medium for any required period. Various modifications of this method have since been frequently employed. It is only necessary to keep cultures of strain *K* 28*c* at a low temperature (till the amoebae have died out) to obtain all the bacteria in them separately.

As this method may be rather slow,† I have used several more rapid procedures which give equally certain results. If a drop of sediment from any culture of strain *K* 28*c* in Ehs medium be streaked on ordinary nutrient agar, and then incubated at 37° C. for 24 hr., it will be found to contain—if the resultant growth be washed off and incubated further in Ehs medium—all the characteristic bacteria (both

* Cf. Dobell (1928, p. 367; 1931, p. 26).

* Cf. Dobell (1927).

† Cysts of *E. histolytica* (strain *K* 28*c*) may live occasionally at 2° C. for as long as 4 months, though amoebae are unable to survive for as many days.

aerobes and anaerobes) of the strain, and no amoebae. A drop of similar material inoculated into and incubated in broth in the same way supplies a similar product. I am not prepared to state that *all* the bacteria in strain *K* 28*c* survive such treatment, but I can say with every confidence that all the essential bacteria survive, and that no amoebae or cysts of *E. histolytica* can do so.

In obtaining the whole flora by this procedure it is important to begin, as stated, with a culture of strain *K* 28*c* which does not contain starch: for starch-fed amoebae often survive in broth for even more than 24 hr. at 37° C., and cysts may not only live but hatch under similar conditions. All bacterial cultures so obtained must, of course, always be carefully checked by subcultivation and microscopical examination before use.

Most cultivated strains of *E. histolytica*—obtained in the usual way, with their naturally concomitant flora—appear at first sight to contain an unanalysable mixture of bacteria. Stained films show a vast variety of forms—especially bacilli and cocci, of all shapes and sizes—both Gram− and Gram+, sporulating and non-sporulating. Only the spiral organisms—spirilla and spirochaetes—are usually absent. (These when present in the original material—intestinal contents or faeces—usually die out and disappear in a few cultural generations.) On plating-out such mixtures, a wealth of diversified colonies are usually obtainable on various media, by aerobic and anaerobic methods of cultivation; and the task of separating and classifying all the constituents seems almost hopeless.

But it is not impossible,* for I have now succeeded in analysing—for all practical purposes—the bacterial constituents of strain *K* 28*c*. It contains—or contained originally—at least fifteen bacterial species or varieties (though others may contain many more), and most of them have now been isolated and studied in pure culture. They will now be briefly described.

(2) Analysis of the bacterial flora of strain *K* 28*c*

I attempted to make a complete bacteriological analysis of strain *K* 28*c* by the usual methods. The mixture was studied microscopically—alive and in variously stained films—and plated out on various solid media which were incubated both aerobically and anaerobically. Pure cultures of the chief recognizable constituents were then isolated in suitable media, and subjected to further study. I have made no attempt to study these bacteria serologically, as this has been unnecessary for my purpose. It has been essential, however, to ascertain their fermentation and other biochemical reactions for the purpose of characterizing and recognizing them. Such investigations have, of necessity, involved much time and trouble, but will not now be described in detail.

It is extremely difficult, in analysing a mixture of this sort, to know when one has come to the end. Many organisms appear different when grown pure, and an accidental contaminant on a plate may easily be mistaken for a genuine but rare constituent. I can only say that the following record represents my ultimate findings after taking all obvious precautions. I am not prepared to state that it is either complete or perfect.

The following therefore is a catalogue of all the bacteria which I have hitherto been able to isolate from strain *K* 28*c*. Exact determination and naming of all species has not been possible, so they are arbitrarily designated by the letters and numbers used in my notes—*A* denoting an aerobe and *N* an anaerobe.

(I) Aerobes

The aerobic bacteria were comparatively easy to study, and may be considered first. They were found to consist of at least eight forms (A^1–A^8).

(i) *Bacterium coli* [=*Escherichia coli*]. There are several distinct varieties of this 'species' in strain *K* 28*c*. When the bacterial mixture is plated out on MacConkey's medium, a number of different types of 'red' colonies can always be found. These are all Gram−, non-sporulating, gelatine non-liquefying, and in every other way typical '*B. coli*'. But they show many different characteristics and some of them 'breed true'. Of the constant types I have gradually come to distinguish at least three, which are now designated A^1, A^2, and A^5. Their chief fermentation reactions and other properties are shown in Table 4 (p. 29) and need not be discussed in detail here. It will suffice to note that A^1 is a 'smooth' (S)* form, with small glossy, convex, sharply-defined colonies on agar; always non-motile; and showing uniform growth in broth.

This form (A^1) usually breeds true, but after prolonged cultivation—and especially after growth in mixtures, and treatment with dyes—it produces rough colonies, in variable proportion, on recovery and replating. The rough (R) forms so obtained differ in no way from A^5, except that they are invariably non-motile—like their parent (S). I have used this variety (A^1) extensively in my experiments, as

* I well remember how, many years ago, Laidlaw and I thought it was. When we first attempted to analyse our original strain *D* (*E. histolytica hominis*), one of us remarked that it apparently contained all the known forms of bacteria! Our plates and stained films certainly gave us this impression.

* I use the terms 'rough' and 'smooth' in the sense of Arkwright (1921).

its smooth and uniform growth offers obvious advantages.

A^5, with which I have also done very many experiments, is apparently a 'rough' (R) form of the same. It is now the prevalent aerobic organism in strain *K* 28*c* and differs from A^1 in forming large irregular, jagged-edged colonies on agar; in being very actively motile; and in forming a precipitate or clots in broth.

A^3 is probably identical with A^5—as I now think. But it is—or may be—intermediate between A^1 and A^5. It usually differs from the latter by being less actively motile, but in fermenting saccharose more rapidly. (A^5 takes usually 2–3 days, A^3 only 24 hr., or less.) When first isolated it often appears on MacConkey plates in flat rough colonies with a dark red centre, or 'bull's-eye', and a paler (sometimes colourless) periphery.

All these strains (A^1, A^3, A^5) of '*B. coli*' are able to split saccharose, and should accordingly be classified as *B. coli communior* [=*Escherichia communior*].

As will be evident from Table 4 (p. 29), all these organisms are identical as regards their fermentations and other properties. These may be modified, however, by certain treatments to which they have been subjected in the course of my experiments. When grown in media containing high concentrations of certain dyes (especially acriflavine and methyl violet) the motile forms lose their motility and all forms grow in long filaments.* These segment into short rods again when normal conditions are restored, and their motility—if originally present—is recovered sooner or later. Treatment with acriflavine has also been found to inhibit—temporarily—their power of forming indole, and on one occasion the same treatment caused A^5 to lose its power of forming gas when fermenting saccharose—acid alone being produced. Similar changes have also been noted when these organisms have been re-isolated from bacterial mixtures into which they were experimentally introduced. They illustrate anew the difficulty of identifying and purifying strains of a notoriously variable species like '*B. coli*'.

(ii) *Bacillus* A^3 and *Bacillus* A^4. These two organisms are invariably present in considerable numbers in strain *K* 28*c*, but are so much alike that they may be described together.

When the *K* 28*c* mixture is plated out on ordinary agar, only two types of colonies can be found in addition to the numerous *B. coli*. These are small, smooth, flat, and soft (A^3) or equally small but rough, slightly rugose, and extremely hard (A^4). The hard colonies are indeed, so tough, that they are often difficult to handle. If touched with a platinum loop they slide about like coins on a plate, and do not readily break up. Both types are whitish or colourless.

When the mixture is plated out on MacConkey's medium only a few white colonies can usually be found. Most of them are A^3. A^4 often does not appear at all—the plates thus giving an erroneous idea of their true frequency.

These organisms are morphologically identical. They are non-motile, non-sporulating bacilli, subject to great variation in size. Indeed they are extremely pleomorphic, and may grow as cocci, coccobacilli, chains of either, and long filaments. Often, when growing as chains of coccoids, they closely resemble streptococci; and when, as often, they grow in clusters of coccal forms, they look like staphylococci. All forms can usually be found in the same culture, though there is some diversity in different media (liquid and solid). It has taken much plating out and careful study to convince me that my cultures were pure.

Neither A^3 nor A^4 can ferment any of the sugars, etc., usually used for identifying intestinal bacteria (see Table 4, p. 29). In this respect, indeed, their characters seem to be wholly negative. They appear to be 'smooth' and 'rough' forms of the same organism, but they always breed true. Neither form has ever turned into the other—despite all the experimental treatments to which they have been subjected.

Apart from the difference of their colonies on agar, A^3 and A^4 can always be readily distinguished by their growth in broth. After 24 hr. incubation at 37° C., A^3 shows a fine uniform growth all through the liquid; A^4 shows an almost clear liquid with a white precipitate at the bottom of the tube.

Both organisms are usually Gram−, but some individuals appear feebly Gram+ in young cultures. This is most clearly seen in films made from mixed cultures. In a pure mixed culture of A^3 (or A^4) and *B. coli*, for example, the slight coloration of the former with Gram's stain is often clearly seen—in contrast with the frankly negative reaction of the latter.*

I have been unable to identify these bacteria with any previously described, so must content myself with designating them by the letters and numbers provisionally assigned to them in my notes.†

(iii) *Bacillus* A^6. Although, as already noted, only three kinds of aerobic bacteria could be found by plating out on ordinary agar or MacConkey's medium, another organism was discoverable—in

* The dye apparently arrests division but not growth—a fact noted many years ago by Vay (1910), and since 'discovered' by many other workers.

* I have sometimes been in doubt as to the Gram-staining of these organisms. Most of my bacteriological colleagues who have studied them say they are Gram−.

† Several of my colleagues, to whom I have appealed for help in this connexion, have likewise failed to give a name to them. All, however, have confirmed the purity of my strains.

very small numbers—when the plating was done on serum-agar. This was labelled A^6. It appeared to live chiefly in the surface film of the liquid in the mixed culture.

A^6 was a small, Gram+, non-motile, non-sporulating diphtheroid bacillus, forming small whitish colonies (on serum-agar) resembling those of a *Streptococcus*. It grew poorly and slowly, when pure, in Ehs medium. Because of its rarity and seeming unimportance, it was not studied in great detail. It was not identified.

(iv) *Bacillus* A^7. This was another rare form, which was never seen on ordinary agar or serum-agar plates, but only made its appearance occasionally when the *K* 28*c* mixture was plated out on blood-agar. It formed very small clear colonies, and was a long slender bacillus, tending to grow in chains and long filaments; non-sporulating, Gram+. Streaked on blood-agar it formed a rather slimy, semi-transparent veil. Like the preceding it was not studied in great detail, and not identified. It also made slow and poor growth, when pure, in Ehs medium.

(v) *Streptococcus* sp. When the original *K* 28*c* mixture was plated out on blood-agar—and on this medium alone—it always showed a number of typical small white colonies of streptococci. These all appeared to be of the same variety—an ordinary non-haemolytic *Streptococcus* [= *Enterococcus*], such as is found in faeces.

This organism was labelled A^8. It was not studied in detail, or used for any of the following experiments. This was because it disappeared from my cultures of strain *K* 28*c* on prolonged cultivation. It was also eradicated from certain lines by treatment with acriflavine, and in both cases its disappearance in no way affected the general properties of the original bacterial mixture. Its presence therefore appeared unimportant.

Streptococci were certainly present, in small numbers, in strain *K* 28*c* from the time of its isolation (in 1926) till the end of 1928 (i.e. for at least 2 years). Thereafter they were seldom seen in any line, and a renewed attempt to recover them recently (October 1943) has again failed completely. My pure cultures were abandoned some 10 years ago, and I have not seen the organism since.

(II) Anaerobes

The anaerobic bacteria in strain *K* 28*c* proved very troublesome to deal with, and I am not sure that I have yet isolated all of them. Probably at least seven distinct species or varieties were originally present, but only three of these (N^2, N^4, N^5) appear to be important constituents of the flora. The supposedly unimportant forms were not studied in great detail.

The bacilli designated N^2 and N^4 are usually* the only spore-forming organisms discoverable in the *K* 28*c* mixture. They were both first isolated pure (by Laidlaw, in 1929) by heating the whole bacterial mixture at 80° C. for 10 min., and then germinating the surviving spores anaerobically on blood-agar, and plating out. Afterwards I obtained these organisms by other methods—all too often, as will appear later.

(i) *Bacillus* N^1. This is a small, Gram−, non-sporulating, non-motile 'diptheroid', forming small transparent colonies on blood-agar. It proved very difficult to isolate pure, and when isolated would not grow on ordinary agar or serum-agar, or in glucose broth or any other medium tried—including Ehs, in which it grew, however, with other organisms. It was fairly abundant on blood-agar plates incubated anaerobically, and was continued on slopes of the same medium. It has not been identified specifically.

(ii) *Bacillus* N^2. This organism is present in considerable numbers in all cultures of strain *K* 28*c*, but is easily overlooked on account of its small size. It is a very slender non-motile bacillus, of variable length, with a tendency to form long bent or angled filaments or chains, with a characteristic appearance. Its spores are minute, highly refractile, and spherical, and are always terminal. Spore-bearing individuals have the 'drumstick' appearance of *B. tetani*, but—being usually longer and somewhat curved—are more comparable with bent round-headed pins.

This organism is strongly Gram+ in young cultures, but completely Gram− in old. Stained films made from pure cultures therefore look impure—the clumps of deeply stained (multiplying) young forms being sharply contrasted with the numerous older and uncoloured individuals lying around them. The appearance of these bacilli in mixtures is often misleading, and varies in different media and under different conditions of cultivation. In pure cultures on blood-agar, for example, very long filaments are usually seen; and these are specially plentiful in the condensation water at the bottom of slopes, where they form a dense, tangled mycelium-like mass—totally unlike the short bacilli ordinarily visible in mixtures grown in Boeck tubes.

Bacillus N^2 grows well on blood-agar, forming very small rugose colourless colonies. It will not grow on ordinary nutrient agar, but grows well in all the media of Boeck-Drbohlav type used for cultivating *E. histolytica* (Ehs, HSre, etc.) under strictly anaerobic conditions. In these media, and also on blood-agar, it does not usually form spores when grown in pure culture, but if the cultures are removed from the anaerobic jar and incubated aerobically, spores are generally formed. When grown in pure mixed cultures, however, with any of the aerobic

* The reason for inserting this qualifying adverb will appear in the sequel.

bacteria (A^1, A^3, A^4, etc.), it grows excellently and sporulates freely under 'aerobic' conditions. I have been unable to identify this bacillus with any previously described and named, and have not noted it in most other strains of *E. histolytica* derived from men or monkeys.

(iii) *Bacillus* N^3. I can say little about this 'species', as I have devoted little attention to it. It is a form closely similar to, but smaller than, N^1, with which it was originally obtained mixed. It could be grown on blood-agar only, but was always present in small numbers on serum plates made from the *K* 28*c*. mixture.

(iv) *Bacillus* N^4. This anaerobe is always present in considerable numbers in cultures of strain *K* 28*c*. It is a slender organism with slightly pointed ends, and should therefore perhaps be classified as a *Clostridium*. It is non-motile and invariably Gram −.

N^4 grows freely on blood-agar and serum-agar, and in all media of Boeck-Drbohlav type (Ehs, HSre, etc.) under anaerobic conditions. On solid media its colonies are minute, round, smooth, and convex—thus easily distinguishable from those of N^2. It sporulates under the same conditions in pure culture, and in pure mixed cultures with the aerobes (A^1, A^3, etc.) of strain *K* 28*c*. The spores are cylindrical and usually subterminal.

(v) *Anaerobe* N^5. This important constituent of the flora is a pleomorphic organism which at first I did not recognize as a distinct species. It occurs in the *K* 28*c* mixture in the form of short clostridia, bacilli, or diplococci (with bluntly pointed ends), and also as chains (of variable length—usually short) resembling streptococci. For this reason it was originally confused with anaerobe N^4 and the *Enterococcus* (A^8) originally present. All—except dead or degenerate organisms in old cultures—are strongly Gram +. N^5 is a strict anaerobe and very difficult to isolate. It will be described in more detail later (see pp. 36 *et seq.*).

(vi) *Bacillus* N^6. This is another small non-sporulating Gram − organism similar to N^1 and N^3. I am not certain that it is really distinct, though it appeared to differ slightly in size and the form of its colonies on blood-agar—the only medium on which it grew. As this bacillus was rare, grew with difficulty, and seemed unimportant and uninteresting, it was not studied in detail.

(vii) *Anaerobe* N^7. This organism occurs in small numbers in the *K* 28*c* mixture, and was the last to be recognized and isolated. It is a small Gram − *Clostridium*—very difficult to isolate, and a strict anaerobe. It grows poorly, and only on blood-agar, in pure culture; but it grows well in pure mixed cultures in Ehs medium with *B. coli* or any of the other aerobes. Under these conditions it also sporulates freely, forming terminal or subterminal round spores.

The size varies considerably, large and small individuals occurring commonly, while sporulating organisms are usually longer than others. Double (half-divided) individuals, and short chains, are frequently seen, and the latter closely resemble certain forms of N^5. Colonies of this organism on blood-agar plates are smooth, convex, and glossy, and indistinguishable from those of N^4 and N^5.

Before describing the experiments made with cultures of several bacterial constituents of strain *K* 28*c*, it will be convenient to summarize the findings recorded so far. The accompanying table (Table 2) therefore lists all the species or varieties which I have as yet succeeded in isolating.

The prevalent organisms—those which form by far the larger part of the flora numerically—are undoubtedly *B. coli* (A^1, A^2, A^5), the unidentified aerobic bacilli A^3, and A^4, and the three anaerobes N^2, N^4, N^5. The remainder appear, from their numbers and properties (to be described presently), to be relatively unimportant.

(3) Properties of individual constituents of the flora. Preliminary inquiries

It is not easy to devise good methods for testing the properties of the various bacterial elements comprised in the flora of strain *K* 28*c*. The obvious way would be to isolate an amoeba or cyst—with a micromanipulator—and attempt to cultivate it further in a pure culture of one or other of the

Table 2. *Bacterial constituents of strain K 28c*

Aerobes	*Anaerobes*
A^1 = *B. coli* (smooth non-motile form)	N^1 = Unidentified non-sporulating 'diphtheroid' bacillus (Gram −)
A^2 = *B. coli* (variable rough form)	N^2 = Unidentified spore-forming *Bacillus* (Gram +)
A^3 = Unidentified pleomorphic bacillus (smooth) Gram −	N^3 = Unidentified bacillus (Gram −) similar to N^1
A^4 = Ditto—rough form	N^4 = Unidentified spore-forming *Bacillus* (Gram −)
A^5 = *B. coli* (very rough motile form)	N^5 = Pleomorphic *Clostridium* (Gram +)
A^6 = Unidentified bacillus (Gram +)	N^6 = Small non-sporulating bacillus (Gram −)
A^7 = Unidentified bacillus (Gram +)	N^7 = Spore-forming *Clostridium* (Gram −)
A^8 = *Streptococcus* sp.	

bacterial constituents. A technique of this sort has, of course, been successfully used with small free-living amoebae (the miscalled '*limax* amoebae'), but it is not easily applied to *E. histolytica*, for the '*limax* amoebae' are aerobes, with thick-walled cysts which can survive desiccation, and they can be plated out and handled roughly at room-temperatures, while *E. histolytica* is a delicate anaerobe which cannot long withstand exposure to air, whose cysts are thin and fragile, and which is sensitive to variation of pH within a narrow range and is instantly killed—at all stages—by drying. After making a number of experiments by such methods of direct isolation, we abandoned them as impracticable. We failed to obtain any 'pure mixed' cultures by such means, though other workers claim more success.

We therefore resorted to indirect methods, and attempted to ascertain the action of particular bacteria by adding them to cultures in excess, or by hatching the partly sterilized cysts in media previously sown with the bacteria to be tested. Both these methods were extensively used and developed in the present researches.

(i) *Factors determining encystation*

I may perhaps recall at this point some of our early results—obtained especially with strain *D* (*E. histolytica hominis*).* From this strain we isolated in pure culture a number of aerobic bacteria, including a bacillus resembling *B. dysenteriae* (Shiga) and two lactose-fermenting bacilli of the *coli-aerogenes* group. These were called *Bacillus* I (lactose non-fermenter) and *Bacilli* III and IV. *Bacillus* I was found to be very favourable for the growth of all strains of *E. histolytica* studied, and also to favour the hatching of cysts. *Bacillus* III appeared to be definitely harmful and unfavourable for hatching. *Bacillus* IV also appeared to be unsuitable for *E. histolytica* in every way (but very favourable for *Endolimax nana*). None of these organisms, moreover, appeared to promote encystation, when present in cultures in excess. The sugar-reactions and other properties of these bacilli (as far as they were ascertained) are repeated for convenience in Table 4 (p. 29).

I attempted to make similar experiments with the bacteria in strain *K* 28*c*. These were studied first of all to test their power to induce encystation. As we have seen, the total flora of *K* 28*c* induced non-encysting strains to encyst, and it therefore became necessary to study its elements separately—to ascertain their individual capacities in this connexion.

* See Dobell & Laidlaw (1926)—particularly p. 309.

[Here it should be noted that the designations *E. histolytica hominis* and *E. histolytica macacorum*, as used by the author in this and previous publications, are not intended to denote subspecies, but merely indicate *strains* of human and simian origin respectively.—C.A.H.]

To do this satisfactorily, it was necessary to experiment with a non-encysting strain of amoebae whose negative behaviour could be guaranteed. Such strains are not easy to obtain, for most 'non-encysting' strains usually form a few cysts sporadically in cultures—even under the most standardized conditions. They are only relatively non-encysting. I therefore selected for study a strain (*DM*) which never at any time, or under any conditions (provided its flora was not altered), had ever been found to produce even a single cyst. This strain must now be briefly described.

Strain *DM* was derived from cysts of *E. histolytica* in the faeces of my tame macaque Mungo (*M. sinicus* ♂), experimentally infected with strain *D* (*E. h. hominis*).* The cysts were passed on 28. iv. 27 (9 days after infection), and carefully washed and concentrated the following day. They were then treated with N/10 HCl at room temperature for 1½ hr., when the suspension was neutralized (with $NaHCO_3$) and the cysts left to settle. The sediment was then inoculated (29. iv. 27) into a tube of HSre + S medium previously sown with *Bacillus* I,† and incubated at 37° C. The cysts hatched excellently, and the resultant culture was continued as strain *DM*.

This strain of *E. histolytica*, therefore, was originally of human origin, but the amoebae had been experimentally passed through a macaque, and its flora consisted of *Bacillus* I, together with the descendants of all those bacteria and spores which had withstood treatment with acid. At the date of the experiment the monkey was also infected with *Endolimax nana*, *Enteromonas*, and *Blastocystis*, but none of these developed in the cultures. Starch-splitting bacteria were also eradicated, so the strain was not treated with acriflavine.

All the bacteria in this strain were not studied in detail, but it was known to contain various spore-forming anaerobes and streptococci (of simian origin). When cultures were plated out on MacConkey's medium they showed an abundant uniform growth of white colonies (*Bacillus* I). *B. coli* was entirely absent—presumably killed by the treatment with HCl. This was the most distinctive bacterial character of the strain.

Two different lines of the strain *DM* were started and maintained in Ehs medium—one with starch, the other without. Both grew excellently. From time to time the starchless substrain was tested for cyst-formation by adding starch, but no cysts were ever found.‡ Strain *DM* appeared, indeed, to be incapable of forming cysts under any conditions or in

* See Dobell (1931), p. 41, for further details of these experiments.

† Cf. preceding column.

‡ Under such conditions, of course, strain *K* 28*c* invariably forms cysts abundantly.

any media (provided its flora was not altered). It grew freely in all my usual media,* and was finally propagated only in Ehs + S (subcultures being made once a week). It was finally abandoned voluntarily on 26. v. 32, after being under continuous cultivation in the amoeboid state for over 5 years (287 cultural generations).

The evidence—from some thousands of cultures—that strain *DM* was a truly non-encysting strain, is to me conclusive. I therefore had no hesitation in accepting it as such in the following experiments.

Before I attempted to study the effects on strain *DM* of individual bacteria from strain *K* 28*c*, I had made numerous tests with the flora of this strain *en masse*—with the usual result (already noted). The method adopted (in this and all similar experiments) was as follows: A tube of Ehs medium was inoculated with the mixed flora of strain *K* 28*c*† and incubated for 24 hr. at 37° C. Into this culture a small inoculum of the amoebae (with their accompanying bacteria, of course) from strain *DM* was then introduced, and the mixture incubated for a further 24 hr. This process was then repeated for several (6–10) generations, when a well-growing strain of amoebae with a flora predominantly that of *K* 28*c* was readily obtained. After further cultivation for a short period—in starch-free medium—it was tested for cyst-formation by transference to a tube of the same medium supplemented by starch (Ehs + S). Abundant cysts were then always formed after 48 hr. incubation—exactly as with strain *K* 28*c* itself. I have made and studied many such substrains of *DM* (usually called strain *DMK*) and always with the same results—and not only of *DM* but of many other strains of *E. histolytica* derived from very varied sources. Every non-encysting strain, when grown thus with the flora of *K* 28*c* as a whole, acquired its properties and became a regularly encysting strain. Such strains, moreover, could always be propagated with ease afterwards—exactly like *K* 28*c*.

After making these preliminary experiments I made a long series of similar tests with the individual species of bacteria isolated from strain *K* 28*c*.‡ The method generally used was that just described, but the testing of the anaerobic bacteria presented difficulties and necessitated some modification of technique. The results were briefly as follows:

When strain *DM* (non-encysting) was grown with *B. coli* (A^1, A^2 or A^5) it behaved almost as though it were supplemented by the whole flora of strain *K* 28*c*. It became an encysting strain—producing good crops of cysts when starch was added to the cultures. But the cysts were not usually as abundant as those obtained by addition of the whole flora, and they never proved viable for such long periods. As a rule they lived for only 2–3 weeks at 2–10° C. (as compared with 2–3 months for strain *K* 28*c*).* All strains, however, formed by adding *B. coli* (A^1, A^2 or A^5) to *DM* grew extremely well in Ehs medium, and could easily be continued.

No other bacteria from strain *K* 28*c* produced the same results. Two other aerobic organisms (A^3 and A^4) proved highly favourable for growth, but were completely negative when tested for encystation. No cysts were ever formed by strain *DM* when supplemented by excess of A^3 or A^4 alone. The two remaining aerobes (A^6, A^7), however, proved highly unfavourable for growth in Ehs medium. When added in excess to strain *DM* they caused its rapid extinction, so that no tests for encystation were possible. Both these organisms were therefore discarded, and not studied further. As already noted (p. 24), no tests were made with the streptococci (A^8) originally present in the *K* 28*c* mixture, as they died out without altering its properties.

The anaerobic bacteria of strain *K* 28*c* were much more troublesome to test, as all cultures had to be made in an anaerobic jar—with other appropriate precautions. I also found it necessary to use 48 hr. cultures (instead of 24 hr.) as insufficient growth occurred in the shorter period. The two spore-forming bacilli (N^2 and N^4) were first tested, and gave entirely negative results. Both, when present in excess, proved harmful to the amoebae in strain *DM*, and on the addition of starch no cysts were ever formed. Both these anaerobes (N^2, N^4) were also tested in another way. As the usual anaerobic technique was so laborious, I tried cultivating them in pure mixed culture with one or other of the aerobes from strain *K* 28*c* (A^3, A^5, etc.). With these they grew excellently under aerobic conditions (the aerobe supplying the conditions necessary for growth of the anaerobe), and as two of the aerobes (A^3, A^4) had been shown to have no effect on cyst-production, mixtures such as $N^2 + A^3$ or $N^4 + A^4$ seemed legitimate substitutes for pure cultures of N^2 or N^4—and were much more easily handled. I made many experiments by such methods, but the results again were negative. Both N^2 and N^4 appeared to be incapable of inducing encystation in strain *DM*.

The anaerobes N^1 and N^3—as already noted—would not grow alone, when pure in Ehs medium,

* It seems worth noting that *DM* was uncultivable in Cleveland's liver-infusion medium. I have had several similar strains, and they disprove the claim that this is the best medium for all strains of *E. histolytica* and successful in 100% of cases.

† Cf. p. 21 *supra*.

‡ The original experiments were made in 1927–9, but many of them repeated later. I here record my final findings from all.

* This is explicable by the supposition that the original flora of strain *DM* contained bacteria which were in some way harmful or unfavourable for the survival of cysts.

but I found that both were easily cultivable by the method just described (i.e. in pure mixed culture with an aerobe, under 'aerobic' conditions). Both were tested in this form, and both proved harmful to the amoebae of strain *DM*. I found it impossible to cultivate them in Ehs medium when either bacillus was present in excess, so no tests for encystation, by the usual method, were feasible. Most of my experiments were made with N^1, as N^3 was more difficult to cultivate, but all were equally negative.

N^5 was not tested in these experiments, as it had not been isolated and identified at the time when they were made (cf. p. 25 *supra*).

The remaining anaerobes (N^6, N^7)—of whose purity I was uncertain—were not tested individually. I cultivated both with an inactive aerobe (A^3), and tested them *en bloc*. They proved highly unfavourable for growth, and never induced encystation, so I did not study them further.

Table 3. *Effects produced by adding known bacteria from an encysting strain* (K 28c) *to a non-encysting strain* (DM) *of* E. histolytica

	Added bacteria	Encystation
	O (control)	−
	K 28*c* (whole flora)	+ + +
	A^1 (*B. coli*)	+ +
	A^2 (*B. coli*)	+ +
	A^3	−
	A^4	−
E. histolytica strain *DM* (non-encysting)	A^5 (*B. coli*)	+ +
	A^6	−
	A^7	−
	N^1	−
	N^2	−
	N^3	−
	N^4	−
	N^6, N^7 (together)	−

It will thus be seen that the anaerobic bacteria in *K* 28*c* appeared to have no action in causing encystation when tested on strain *DM*. Indeed, they even appeared to be antagonistic in this respect, for I made a series of experiments with some of them which showed this clearly. For example, *DM* grown with A^5 alone produced fair crops of cysts, but grown with A^4 or N^2 it produced none. When grown with A^5+N^2 or A^5+N^4, however, few or no cysts were formed on testing. The anaerobes thus appeared to inhibit the cyst-inducing action of the accompanying aerobe (A^5).

I must add that I also obtained some anomalous results with mixtures of the various pure cultures just described. I made many experiments with these, and discovered that the combinations A^3+N^4 and A^4+N^4, when added to strain *DM*, occasionally produced a few cysts—always very scarce, but normal and viable.* As neither N^4 nor A^3 nor A^4 alone appeared to possess this property, these findings were perplexing. At first I imagined that the irregular results were due to accidental contamination of the atypical cultures, but on repeating the experiments, and checking them by plating out and other tests, I satisfied myself that this was not so.

From all these experiments only one definite conclusion could be drawn. All the anaerobic bacteria in strain *K* 28*c* were inactive as regards encystation, and most of them were even harmful for development. The aerobes, tested severally, proved equally inactive with one striking exception—*B. coli*, all varieties of which (A^1, A^2, A^5) promoted growth, induced encystation, and generally favoured the completion of the life history of the amoebae. To summarize these results of much laborious experiment, I have cast them into the accompanying Table 3, which shows them at a glance.†

These results thus appeared to solve my main problem. Of all the bacteria present in strain *K* 28*c*, one species only—*B. coli*—was capable of inducing encystation. It appeared to be the essential ingredient in the mixture. At least three different varieties of this bacillus were present—all possessing similar properties—so it seemed probable that other varieties, from different sources, would behave in the same way. I therefore isolated and tested several other strains of '*B. coli*'. Five of these were particularly studied, and must now be briefly described. They were known as L^1, L^2, Δ^1, Δ^2, and DKB^2.

At the time when these experiments were made (1931) I had another strain of *E. histolytica* which encysted spontaneously and regularly like *K* 28*c*. On testing the total flora from this strain (*RF*) I found that it would induce encystation in strain *DM* equally well. When plated out, likewise, it was found to contain '*B. coli*' in abundance, and I isolated and tested two varieties of these especially. They were labelled L^1 and L^2, and their general characters are summarized in Table 4 (p. 29). (It will there be seen that they differed in various ways from A^1, A^2 and A^5.)

Strain *RF* of *E. histolytica* was derived from the faeces of my tame macaque Rosa (*M. rhesus*), who had previously been infected experimentally with a human strain (*D*) of the amoeba. The amoebae were therefore ultimately of human origin, but their accompanying flora was simian. The strain was isolated on 14. xi. 30, and voluntarily abandoned at the 59th serial subculture on 28. vii. 31. In general, it behaved like *K* 28*c* and produced cysts freely under the same conditions.

The bacilli called L^1 and L^2—isolated from *RF*—were similar, but both differed from 'typical *B. coli*',

* This was proved by cytological study and hatching *in vitro*. No animal tests were made.

† For the bacteria denoted by the symbols A^1–A^7 and N^1–N^7, consult Table 2 (p. 25).

Table 4. *Biochemical characteristics of the various aerobic bacteria*

	Dextrose	Laevulose	Galactose	Lactose	Maltose	Saccharose	Raffinose	Mannitol	Dulcitol	Indole	V.-P. reaction	Gelatin	Litmus milk	Motility	Gram	Salicin	Starch	Approximate name
Bacillus I	A	–	A	–	–	–	–	–	–	–	–	–	C	–	–	–	–	
Bacillus III	AG	O	O	AG	O	AG	O	AG	–	–	+	–	AC	–	–	O	–	*B. lactis aerogenes**
Bacillus IV	AG	O	O	AG	O	AG	O	AG	–	–	–	–	AC	+	–	O	–	
Bacillus A[5]	AG	AG	AG	AG	AG	AG	AG	AG	AG	+	–	–	AC	+	–	–	–	*B. coli communior* (R)
Bacillus L[1]	AG	AG	AG	AG	AG	AG	–	AG	AG	–	–	–	AC	+	–	–	–	
Bacillus L[3]	AG	AG	AG	AG	AG	–	–	AG	–	–	–	–	AC	–	–	–	–	
Bacillus Δ[1]	AG	AG	AG	AG	AG	–	–	AG	AG	+	–	O	AC	+	–	AG	–	*B. coli communis* (?)
Bacillus Δ[3]	AG	AG	AG	AG	AG	–	–	AG	AG	–	–	–	AC	+	–	AG	–	
Bacillus DKB[3]	A	A	A	A	A	A	A	A	A	–	–	–	AC	–	–	–	–	*B. coli anaerogenes*
Bacillus X	Alk	O	O	Alk	O	Alk	O	Alk	Alk	–	O	–	O	+	–	O	–	*B. faecalis alkaligenes*†
Bacillus CW[1]	AG	–	AG	–	–	–	–	–	–	–	O	+	Alk no C	+	–	–	–	*B. proteus*‡
Bacillus A[3]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	
Bacillus A[4]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	
Bacillus A[2]	AG	AG	AG	AG	AG	AG	AG	AG	AG	+	–	O	AC	+	–	–	–	*B. c. communior* (R) [= A^5?]
Bacillus A[1]	AG	AG	AG	AG	AG	AG	AG	AG	AG	r	–	O	AC	–	–	–	–	*B. c. communior* (S) [= A^5]
Bacillus CL[1]	AG	AG	AG	AG	AG	–	–	AG	–	+	–	O	AC	–	–	–	–	*B. c. communis* (R)
Bacillus CL[3]	AG	AG	AG	AG	AG	–	–	AG	–	+	–	O	AC	–	–	–	–	*B. c. communis* (R)

* (= *Aerobacter aerogenes*).
† (= *Alkaligenes faecalis*).
‡ (= *Proteus vulgaris*).

in not forming indole. L^1 was motile, L^2 was not. The latter was also very rough, and formed a dense, slightly mucoid sediment when grown in broth.

Two other strains of '*B. coli*' I isolated from a sample of normal human faeces. These were known as Δ^1 and Δ^2. Neither fermented saccharose; both were motile; only the first formed indole. (For other characteristics see Table 4, p. 29.)

The fifth variety of '*B. coli*'—known as DKB^2—I isolated from a substrain of *E. histolytica* derived from my old strain *D* (*E. h. hominis*),* in which it must originally have been present. Its characteristics are shown in Table 4 (p. 29), and it will there be seen that it differed from all my other strains by its inability to produce gas in the fermentation tests employed.

All these five varieties of '*B. coli*'—after careful typing—were tested with strain *DM* in the same way as the bacteria from strain *K* 28*c*. They all behaved like the '*B. coli*' variants derived from this strain. When added in excess they caused encystation, and produced encysting strains of amoebae which could be continued *ad libitum* (like strain *K* 28*c*).

Nevertheless, I found that the strains produced in this way were not all exactly alike. Some formed cysts more copiously than others, and only one—that made with L^2—was fully equal to strain *DMK* (made by adding the total flora of *K* 28*c* to *DM*). Moreover, the cysts produced by these strains were seldom as long-lived as those of strain *K* 28*c*. As a rule they survived for only 2–3 weeks at 5–10° C., though appearing normal and typical in every other respect.†

The results of all these experiments are briefly summarized in Table 5 (cf. Table 3, p. 28; and see Table 4, p. 29, for the differential characters of the strains of '*B. coli*').

Table 5. *Effects produced by adding various strains of* 'B. coli' *to a non-encysting strain* (DM) *of* E. histolytica

Added bacteria		Encystation
E. histolytica strain *DM* (non-encysting)	O (control)	–
	B. coli L^1	++
	B. coli L^2	+++
	B. coli Δ^1	++
	B. coli Δ^2	++
	B. coli DKB^2	++

It is clear, from the foregoing experiments, that '*B. coli*' exercised a determining influence upon the production of cysts in strain *DM*. This influence might obviously be due to more than one factor—for example, the bacteria themselves (which the amoebae eat), or some of their products (produced by growth in the medium). Some of my attempts to elucidate this problem may therefore be noted here.

In the first place, I tried to induce encystation in strain *DM* by adding excess of dead '*B. coli*' (instead of living) to cultures of this strain. Experiments were made with several varieties (A^5, L^2, etc.), but all gave negative results. The amoebae ate the dead bacteria readily, but it did not cause them to encyst.* The dead bacteria used in these experiments were killed by heating pure cultures suspended—in an open tube—in water or Ringer's fluid. After many trials I found it necessary to heat some strains (especially A^5, a very rough and clotted form) for as long as 45 min. at 75° C. to ensure sterility. Suspensions heated for 30 min. at 70° C. seldom proved completely sterile when carefully tested.

I made a further series of experiments in which the dead bacteria were obtained by growing pure cultures for various times in Ehs medium, then removing the liquid and sterilizing it in the autoclave, and using the killed suspension (instead of dilute horse-serum) over egg slopes. Media prepared in this way and tested with strain *DM* never caused the amoebae to encyst. Several strains of *B. coli* were thus tested (especially L^2, which when alive was particularly effective), and also the total flora of *K* 28*c*. All appeared to be harmful—retarding or preventing growth of the amoebae.

To test the effects of bacterial products separately, I added sterile filtrates†—of various ages—of cultures of '*B. coli*' (especially A^5 and L^2) to strain *DM*. Again the results were wholly negative. No cysts were ever found in cultures of *DM* to which the filtered products of growing *B. coli* in Ehs medium were added.‡ As a rule such filtrates merely proved harmful.

I may add here that I have tested, from time to time, not only the bacteria belonging to strain *K* 28*c* but also a number of others isolated from other strains. The results, in general, have no present interest, but I may record that one organism occurring not infrequently in dysenteric stools—*B. faecalis alkaligenes*, which we isolated originally

* This was in agreement with earlier experiments made with the total bacterial flora of strain *K* 28*c*. The bacteria of this strain, when killed by heat and added to non-encysting strains of *E. histolytica*, have never caused encystation—in striking contrast with the effects of living bacteria. Usually, the dead organisms proved unfavourable or even harmful for growth.

† In all these experiments the filtrations were kindly made for me by my colleague, Dr W. J. Elford. The bacteria were filtered through his 'Gradocol' membranes with an average pore diameter of 0·7μ under positive pressure (nitrogen).

‡ In agreement with previous (but unrecorded) experiments with the total flora of strain *K* 28*c*. Filtrates of this strain, when added to non-encysting strains of *E. histolytica*, never induced encystation.

* Cf. p. 26 *supra*.

† Cf. 4th footnote on p. 27.

(under the name *Bacillus X*) from strain *D*—* appeared to be definitely harmful. When added in excess to cultures of *E. histolytica* it does not promote growth or induce encystation, but apparently inhibits both. It thus seems to play no essential role in the life history of the amoeba.

All these results—taken together—thus appeared to prove that the essential constituent of the flora of strain *K* 28*c* was *B. coli*. When any variety of this organism was added to a non-encysting strain of *E. histolytica* (*DM*) it induced encystation in the same way as the total flora.† Moreover, the cyst-producing properties resided somehow in the living bacteria—since they were seemingly absent from dead organisms or filtrates containing their products. Other experiments made simultaneously by a different method appeared to confirm this conclusion, and some of these must now be described.

(ii) *Factors determining excystation*

It has been noted already that the properties of the bacterial flora can be investigated by hatching the cysts of *E. histolytica* in cultures of known bacteria, and cultivating the mixture of organisms further. If sterile cysts could be obtained for this purpose, and pure cultures of bacteria were used, 'pure mixed' cultures—of known constitution—could then be studied, and the properties of individual bacteria could be accurately determined. Some preliminary results obtained by this method have already been described.‡ As will be evident, it enables one to study not only the action of various bacteria in inducing excystation, but also their effects on the growth of the amoebae and—by further cultivation—their effects on encystation. Unfortunately, it is extremely difficult to obtain sterile cysts of *E. histolytica*. They are always formed—whether in the intestine or in the culture-tube—in a dense mixture of bacteria, and to free them from these mechanically (by repeated washing) or by the action of antiseptics is not at all easy. Indeed, the cysts are usually found to be less resistant, or more easily killed, than the accompanying organisms which it is desired to remove.

In our early experiments we attempted to sterilize the cysts (from faeces or cultures) by treating them with hydrochloric acid. We found that they could survive in this in concentrations and for times which were fatal for most of the accompanying bacteria. But further careful trial of this method showed me that the results were inconstant and unreliable, though it furnished useful indications. After much experimentation and innumerable failures—which it is not necessary to describe—I finally hit upon a simple method for freeing the cysts of *E. histolytica* from all accompanying bacteria without injuring them. This was by the use of strong solutions of acriflavine, to which ripe cysts are relatively impermeable,* though the dye will kill all concomitant amoebae and bacteria. I devised this method some 15 years ago, and have used it extensively with complete success ever since.

The method finally adopted—and here employed especially with strain *K* 28*c* though it is generally applicable—is as follows:

A good crop of cysts (*E. histolytica*, strain *K* 28*c*) is obtained in Ehs + S medium in the usual way. The culture is then removed from the incubator (37° C.), and left to cool to room temperature. The liquid is then pipetted off, and the sediment—containing cysts, amoebae, bacteria and starch—is transferred to a test-tube containing 5–10 c.c. of sterile water (or other suitable liquid). In this the cysts, etc., are allowed to settle, and the supernatant liquid again pipetted off. This process is repeated several times, until the sediment contains a clean concentration of cysts (with their concomitants). These are then transferred to a test-tube containing 5–10 c.c. of 0·1 % acriflavine solution (1 part in 1000), and put in the refrigerator. The suspension is then kept there till all the bacteria are killed, which may require a week or more. At the end of this time the sedimented cysts are removed and washed, as before, several times. When they are clean, and the washing liquid is almost colourless, they are ready for experimental hatching. To ensure success, attention must be paid to certain details, and I therefore add the following notes:

(1) All operations must be carried out under *strictly aseptic conditions*. The washing liquid, and even the acriflavine solution, should be sterilized in the autoclave. (Boiling is not always sufficient to ensure the absence of air-borne spores of bacteria or moulds.)

(2) The *washing liquid* may be water (tap or distilled), normal saline (0·75 % NaCl), or Ringer's fluid; all give good results. But I generally use *half-strength* Ringer's fluid (Ringer diluted with an equal volume of distilled water), which I have found most satisfactory and recommend.

(3) A *good acriflavine* should be used, and the solution made up accurately. The dye may be dissolved in water or Ringer's fluid. I have used throughout a sample of the dye manufactured by Messrs Boots (about 1918), and generally make my solutions in sterile distilled water. It is best to make up small quantities at a time, and keep the solution in the dark.

* See Table 4 (p. 29).

† [But see footnote on p. 33.—C.A.H.]

‡ Cf. Dobell & Laidlaw (1926).

* Before they are mature, the cysts of *E. histolytica* have more permeable walls: consequently uninucleate and binucleate cysts are easily killed (and stained yellow) by comparatively weak solutions of acriflavine.

(4) The *temperature* at which the dye is allowed to act on the cysts and accompanying bacteria is very important. Any temperature between 2° and 10° C. gives satisfactory results, but at higher temperatures the cysts may be killed on prolonged exposure. I now always treat cysts at a constant temperature of 2° C., which I have found most suitable.

(5) The *time* for which the acriflavine must act, at 2–10° C., to kill all concomitant bacteria without injuring the cysts is also most important, but depends upon the species of bacteria present. *B. coli* is especially resistant to the dye, and may survive in strong acriflavine for many days, though under such conditions all the other bacteria in strain *K* 28*c* are rapidly killed. As a rule suspensions of cysts are free from all living bacteria in a week, but in the course of my work I have found *B. coli* still alive—on isolated occasions—after 8, 10, 11, and (once only) even after 13 days. It is therefore advisable to leave the cysts in acriflavine for 14 days to make sure of their sterility. The cysts themselves will usually live in 0·1% acriflavine at 2° C. for at least 3 weeks.

In testing flavine-treated suspensions for bacterial sterility, it is necessary to wash the stained bacteria thoroughly before smearing them on agar, etc., for the dye has a strong bacteriostatic action, and when present in the inoculum may give 'sterile' cultures which more careful study may prove to be really alive. Suspensions of living cysts 'sterilized' in the above manner are found to be completely free from all living bacteria when tested by aerobic methods of cultivation (broth, agar, etc.). But when studied more carefully by anaerobic methods it is invariably found that the cysts are not truly sterile. They are accompanied by the living spores of any anaerobic bacteria which happen to be present, for these are far more resistant to acriflavine—and all other known chemical and physical agents—than the cysts of *E. histolytica*. Consequently, cysts of strain *K* 28*c*, when 'sterilized' as just described, are always accompanied by living spores of the anaerobic bacilli called N^2 and N^4.

This applies (*mutatis mutandis*) to all other cysts of *E. histolytica* however obtained—whether from cultures or from faeces. In my experience spore-forming anaerobic bacteria are invariably present in such material, and they form their spores under the same conditions as those which cause the amoebae to encyst. This is the chief obstacle in the way of obtaining really sterile living cysts of *E. histolytica*—free from all concomitant bacteria and their spores. Partially sterilized suspensions of cysts—containing cysts and spores only—can, however, be used for a great variety of experiments, and their study has yielded many interesting results. Material containing cysts of *E. histolytica* and the spores of only two known species of bacteria is much simpler to investigate than the *K* 28*c* strain with its fifteen or more concomitant organisms.

Working with such initial material I have now made and studied many strains of *E. histolytica* with a simplified flora consisting of two or more species of bacteria only—all of which were known. I shall describe some of the most interesting presently.

To avoid repetition I may say here that all these simplified strains were made with pure cultures of bacteria—carefully isolated and tested previously—and that the composition of the mixtures was repeatedly verified by microscopical examination (of both living and fixed-and-stained organisms), and by frequent plating out and recovery in pure culture of the various components. The usual bacteriological technique (both aerobic and anaerobic) was employed, and all 'synthetic' strains described were cultivated for sufficient time (weeks or months) to make sure of their composition and properties.

If carefully washed suspensions of living cysts (*E. histolytica*, strain *K* 28*c*), containing spores of the anaerobic bacilli N^2 and N^4, but no living bacteria or amoebae, are inoculated into any of the usual media and incubated aerobically at 37–38° C., no cultures of any of these organisms are ever obtained. The tubes always appear to be completely sterile. After 24 hr. all the cysts are dead, though the ungerminated bacterial spores are still alive—as can be proved by suitable further tests. Moreover, if such cultures are incubated anaerobically—in a jar filled with hydrogen—the cysts likewise do not hatch. They die, as before, but the spores germinate and produce a pure mixed culture of N^2 and N^4. This result greatly puzzled me at first, so I have repeated the experiment many times—always with the same results.

Excystation of the amoebae can be very easily obtained, however, by incubating a 'sterile' mixture of cysts and anaerobic spores with a suitable aerobic bacterium. All the chief aerobic bacteria in strain *K* 28*c* have proved suitable for this purpose. In order to make the cysts hatch *in vitro* it is only necessary to incubate them aerobically with *B. coli* (A^1, A^2 or A^5) or with either of the bacilli called A^3 and A^4. Excellent cultures—in no way inferior to those obtained from *K* 28*c* itself, with its whole bacterial flora—can be readily obtained in this way.

All the other strains of '*B. coli*' previously mentioned (L^1, L^2, Δ^1, Δ^2, DKB^2, etc.—see Table 4, p. 29) have also been carefully tested, and produce the same result. In addition to these, I have likewise studied a number of other aerobes obtained from other strains of *E. histolytica*—including '*Bacillus* I' (Table 4, p. 29) and *B. proteus* (isolated from my strain *CC*, a naturally encysting strain of *E. h. hominis* derived from a patient with amoebic dysentery). Any one or these bacteria, when inoculated into a mixture of cysts and anaerobic spores and incubated

in any of the usual media aerobically at 37° C., induces excystation and produces an excellent culture of amoebae. Moreover, it is not necessary to use intestinal bacteria. The same result can be obtained with pure cultures of other aerobes—such as *Staphylococcus* or *B. prodigiosus*. In short, a wide variety of aerobic bacteria can be used to cause excystation. Their action is not specific—as it appeared to be in the case of encystation (where *B. coli* alone proved efficacious).

If 'sterilized' cysts of *E. histolytica* (strain *K* 28*c*) are incubated with dead aerobic bacteria (*B. coli*, etc.) instead of living, they do not hatch—nor do the accompanying spores of anaerobic bacteria germinate. The cysts merely die at 37° C., and the cultures—if incubated aerobically—appear sterile. Furthermore, if sterile filtrates of *B. coli* cultures (grown in Ehs medium) are substituted for the live bacteria, the same result is obtained, and a combination of dead bacteria and filtrate of a living culture is equally ineffective. To cause the cysts to hatch it is necessary to incubate them at 37° C. with living *B. coli* (or other suitable aerobes) in any of the usual media.

The foregoing experiments—first made many years ago, and since frequently repeated and varied—appear to show that living bacteria are needed for the hatching of cysts; but this interpretation does not amount to a demonstration, as further work has proved. What the experiments really show is that the *conditions* created by the presence of certain living aerobic bacteria (such as *B. coli*) are necessary for excystation when cultures are incubated aerobically. The products of these organisms, or their dead bodies, do not supply the essential factors.

Before proceeding further, it will be convenient to summarize the main results of the preliminary experiments recorded in this section (p. 31). They indicate that the various constituents of the complex bacterial flora of strain *K* 28*c* are not all essential for its development *in vitro*. Most of the anaerobes appear to be unnecessary, or even—by themselves—harmful, but the chief aerobes (*B. coli* in its various forms, and the bacilli called A^3 and A^4) exercise a profound influence on the life history. *B. coli* supplies a factor (or factors) determining encystation, and all three 'species' are able to induce excystation. All three, moreover, can severally supply the conditions necessary for growth and division.*

It is clear that the part played by each of these bacterial 'species' can be more accurately defined by studying simpler mixtures, and this line of inquiry was therefore followed in subsequent experiments.

(4) Production and properties of simplified strains

It will now be evident that the methods devised for obtaining 'sterilized' cysts of *E. histolytica* (strain *K* 28*c*) and causing them to hatch *in vitro*, supply a means for producing strains accompanied by a greatly simplified flora. I have made and studied many such strains during the last 15 years, and some of these will now be described.

Series 1

(i) *Strains with only three bacteria*

When 'sterilized' cysts—prepared by the method described above (p. 31)—are incubated with a suitable aerobic bacillus, they produce a culture containing amoebae, the aerobe, and the two anaerobes N^2 and N^4 (whose spores are always present with 'sterilized' cysts). A strain of amoebae accompanied by only three bacteria can thus be easily obtained.

Since *B. coli* appeared to be the most important aerobic constituent of the whole *K* 28*c* mixture, my first experiments were made with this organism. All varieties (A^1, A^2, A^5) produced excellent cultures of amoebae, which could be readily propagated indefinitely. Many strains made in this way have been studied in detail. They were generally labelled KA^1, KA^5, etc.—according to the variety of *B. coli* employed.

Strain KA^5—one of many kept for various periods—may be taken as a typical example. It was made, as already indicated, by hatching cysts of *E. histolytica*, treated with acriflavine, in the presence of *B. coli* (A^5). In the primary culture the spores of the accompanying anaerobes (N^2, N^4) also germinated, and consequently the strain ultimately produced consisted of *E. histolytica* accompanied by three bacteria only—*B. coli* (A^5) together with the anaerobes N^2 and N^4. All organisms grew freely in this association, in all the media favourable for strain *K* 28*c*. From my previous experiments with *B. coli*, I expected that strain KA^5 would encyst under the same conditions as the original strain (*K* 28*c*), but to my surprise I found that this was not so. When KA^5 was grown in Ehs medium—even for weeks or months—and then transferred to the same medium supplemented with starch, it consistently refused to form cysts. Various other media were also tried, but always with the same negative results.

Other strains were then prepared and tested using various strains of *B. coli* (A^1, L^3, Δ^2, etc.), but with the same disappointing outcome. It became clear that

* [Since the author's subsequent investigations show that *B. coli* does not induce encystation, it is obvious that he had not revised this paragraph. It therefore records his views at the time when it was originally written, but is in contradiction both to his statements in the next column and on p. 34, and to the findings reported on pp. 34 *et seq.*—C.A.H.]

all strains in which the amoebae are accompanied only by *B. coli* and the anaerobes N^3 and N^4 are non-encysting strains—at the time a puzzling and unexpected discovery. Although I had little hope that they would behave differently, I also prepared a number of other strains in which the *B. coli* was replaced by another aerobic bacterium: KA^3 (consisting of amoebae accompanied by A^3 and N^3 and N^4 only), KA^4 (=amoebae+A^4 and N^3+N^4 only), *KB* (=amoebae+*Bacillus* I+N^3+N^4 only), *KS* (=amoebae+*Staph. aureus*+N^3+N^4 only), etc., etc. All these strains, when carefully cultivated and tested, proved to be non-encysting strains.

As these results did not agree with those previously described, I imagined that the anaerobes (N^2 or N^4, or both) might perhaps inhibit the cyst-inducing power of *B. coli*, so I made a further series of experiments in an attempt to obtain a strain of amoebae accompanied by *B. coli* alone—without any anaerobes. These will now be briefly described.

(ii) *Strains with only two bacteria*

It might seem easy to convert a simplified strain such as KA^5—containing only amoebae and the three bacteria A^5, N^3, and N^4—into still simpler strains comprising only one or two of its bacterial elements. But in practice it has proved extremely difficult.

I began by attempting the separation by mechanical methods.

[*At this point Dobell's manuscript ends and the account is taken up by Dr R. A. Neal.*—C.A.H.]

When Dobell's manuscript was examined, a number of pages were found folded and obviously discarded. The contents of these sheets proved to be contradictory to the final text, in that the factor promoting encystation of *E. histolytica* was attributed to *B. coli*. From a close inspection of the type of paper and of the colour—and hence the age—of the ink employed in writing different portions of the manuscript, and by checking this against the daily entries in Dobell's laboratory notes, it became evident that this paper was written at different periods. From the available data the following sequence of events was established.

The writing of the manuscript was commenced some time before 1943, prior to the isolation of the pure strains $K+A^1$ and $P+A^1$, when Dobell felt certain that the presence of *B. coli* was necessary for the encystation of *E. histolytica*. However, when the pure mixed strains were prepared and examined, it became obvious that this conclusion was wrong.

Starting in October 1943, he carried out further investigations, which culminated in the isolation of the anaerobe N^5 and the demonstration of its remarkable properties. It is probable, therefore, that sometime in 1949 he began to revise the part of the manuscript which had already been written, discarding the pages which were found folded. He then proceeded to write the later results of his observations, but unfortunately died before the text was brought to an end.

As the results are of very great interest, it was decided to try and complete the paper on the basis of Dobell's laboratory note-books. This entailed the perusal of all the entries in thirty-one closely written note-books, with the view to determining the properties of many strains that were synthesized by him. Fortunately, Dobell had made copious notes, from which it was possible to reconstruct his experiments. In general, only those experiments which gave definite positive results are recorded below. This was felt to be the safest procedure, in spite of the fact that it necessitated the omission of a great many experiments. Since Dobell himself had not completed the revision of his manuscript, it will be seen that some of the results described below conflict with Dobell's earlier conclusions. However, it was thought best to preserve Dobell's original text without alteration.

As there are many disadvantages in completing a manuscript posthumously, the publication of this work has been delayed until the methods used by Dobell for preparing pure mixed strains of *E. histolytica* could be repeated and verified. These confirmatory experiments are described in a separate paper (*Parasitology*, **42**, 40).

R. A. NEAL

Attempts to separate by mechanical means the mixture of bacteria and amoebic cysts present in strain *K* 28*c* were made by treating the mixture with 'mitogenetic rays' and also by allowing the mixture to fall through a column of Ringer's solution, but no separation was obtained by either of these methods. An attempt was then made to free the flavine-treated cysts from spores by exposing the mixture to an atmosphere of pure oxygen, but this did not inhibit the growth of the spore-bearing anaerobes. One strain, *Kf* (=No. 2 in Table 6) (and similar other lines), was made by hatching the cysts in the presence of A^5 and then transferring the amoebae to a medium consisting of an egg-slope (E) covered with very little fluid (hs). In this medium the amoebae and A^5 grew well, but all the spore-bearing anaerobes, except N^2, were inhibited and eliminated. Attempts to remove the remaining N^2 by washing the amoebae were unsuccessful, though this species of anaerobe was so greatly reduced in numbers that—with less careful study—the strain would have passed for a culture of *E. histolytica* growing only with A^5.

Other strains, *KC* and *KA* (Nos. 3 and 4 in Table 6) were made by hatching the flavine-treated cysts in a medium which only supported a poor growth of A^5. The degree of anaerobiosis was not sufficient to reduce methylene blue, but allowed the cysts to hatch and the amoebae to develop. In these experiments, all anaerobes were eliminated except N^2 which grew poorly. Strains *KC* and *KA* appeared to consist of amoebae and A^5 only, but, as in the case of *Kf*, N^2 was always recoverable by suitable methods. All these strains, *Kf*, *KC* and *KA*, the bacterial flora of which consisted of A^5 and N^2, grew well in all the usual media, but would not form cysts.

(iii) *Strains with only one bacterium*

As it was found impossible to remove the spore-bearing anaerobes by mechanical methods, attention was turned to the dyes acriflavine and gentian violet. Many experiments carried out with acriflavine in attempts to remove the anaerobes were without success, but this result was eventually obtained with gentian violet. The method employed was as follows.

Cysts of *K* 28*c* were treated with flavine, as described above, in order to kill the aerobes, and then induced to hatch in the presence of pure A^1. This strain, which consisted of amoebae, all the spore-bearing anaerobes and one aerobe A^1, was called *KA3N* (No. 9 in Table 7), the properties of which are described below (p. 36). Here it will suffice to say that the strain formed cysts with great ease. Cysts of strain KA^1 were treated with flavine and hatched in HSre + S medium which had been previously seeded with a pure culture of A^1. After the amoebae were observed to be growing normally, they were subcultured into HSre + S medium seeded with A^1. Prior to inoculating the amoebae, 1–4 drops of a 1% solution of gentian violet, or methyl violet, was added to the medium.* This prevented the multiplication of the bacteria but allowed the amoebae to grow. The amoebae were very resistant to these dyes and even ingested dead bacteria stained purple with the dye. The amoebae were subcultured daily in this manner for thirty-eight subcultures. In the first six subcultures two drops of 0·1% acriflavine were added in addition to gentian violet. The bacteria growing with the amoebae were examined at intervals, and on 18 February 1944 only A^1 was found. But later, after subculturing in the normal manner in HSre + S without any added dye, it was observed that the anaerobe N^7 was still present. This necessitated further treatment with gentian violet, after which the pure strain was finally obtained on 24 November 1945. This conclusion was checked and amply confirmed on many subsequent occasions.

The amoebae of the new strain $K + A^1$ (No. 1 in Table 6), associated with one bacterium A^1, grew excellently in all the usual media both with and without rice starch. It was maintained in HSre + S medium in which it lived regularly for 7 days and, on isolated occasions, for as long as 15 days. It also grew easily in horse-serum diluted with Ringer's solution (hs + S) and provided an improved method for *in vitro* testing of amoebicidal drugs (see Dobell, 1947). In spite of the abundant growth of amoebae, this strain never formed cysts. This conclusion was reached after careful testing and is in marked contrast to the parent strain, *KA3N*. Strain $K + A^1$ was finally abandoned on 20 November 1949, that is after 48 months of cultivation with A^1. Although during this time cysts were never produced, by adding the total *K* 28*c* flora good viable cysts were again formed.

In order to confirm these results another strain of *E. histolytica* growing only with A^1 was prepared. Strain *PC* was isolated from a human case of amoebic dysentery, and the cysts of this strain were treated with flavine. The cysts were then hatched in HSre + S medium which had been previously seeded with A^1. The strain, which consisted of amoebae, A^1 and an unknown mixture of spore-bearing organisms, was then treated with gentian violet, as described above for $K + A^1$. The amount of dye was approximately 1 ml. of a 0·1% solution of gentian violet, which was added to from 1 to 1·5 ml. of Ringer-egg albumen (re) liquid covering the slope. After twenty subcultures with gentian violet, on 6 February 1945 a strain was obtained in which the amoebae were growing with pure A^1; it was designated $P + A^1$ (No. 17 in Table 6). Many similar strains with identical properties were also prepared by a different method described below (p. 37). Strain $P + A^1$ grew readily in all the usual media and lived regularly for 7 days and occasionally for 12 days. Cysts were never formed when tested in the usual way, but by adding the total *K* 28*c* flora viable cysts were produced.

Pure strains were also prepared by hatching sterile cysts of strain PNA^3 (see below, p. 37) with A^3 (strain $P + A^3$, No. 19 in Table 6) and *B. proteus* (strain $P + p$, No. 18 in Table 6). The amoebae grew poorly but regularly in Ehs + S medium and could have been cultivated indefinitely but would not encyst. Many attempts were made to hatch sterile PNA^3 cysts with *Staphylococcus aureus*, but this species of bacteria would not support the growth of amoebae.

In view of these results it is certain that although A^1 (*B. coli*), A^3 and *B. proteus* are capable of supporting the life of the amoebae indefinitely, they will not induce encystation.

(iv) *Various synthetic strains*

Other synthetic strains were prepared by hatching 'sterile' flavine-treated cysts of *K* 28*c* in media which had been previously seeded with pure cultures of various aerobic bacteria. The cysts, together with the concomitant spore-bearing anaerobes, were hatched in the presence of *B. proteus* CW^1 (strain *KP*), *B. prodigiosus* (strain *Kp*), and *S. aureus* to which A^5 was added at the first subculture (strain *KSA*).

Strains *KP*, *Kp* and *KSA* grew readily in Ehs and HSre + S, and formed cysts easily in Ehs + S, though in fewer numbers than the parent strain *K* 28*c*. These cysts were viable and could be hatched in the normal way. Strain *KSA* grew poorly in Ehs but

* In this series of experiments, Dobell did not keep a detailed record of the amount of dye used, or of the final dilution in the medium.

better growth was obtained when starch was added to the medium (Ehs + S), cysts being formed in small numbers.

The 'sterile' cysts of *K* 28*c* were hatched without a supplementary aerobic bacterium by adding two drops of 10% thioglycollic acid to the medium. This strain *KN* (amoebae + anaerobes) grew very poorly and was troublesome to propagate. It did, however, form cysts but the crop was poor and the cysts did not hatch.

Other strains were synthesized by adding pure cultures of one or more bacteria to $K+A^1$ or $P+A^1$ (see Table 6). All these strains grew easily in the usual media but constantly refused to form cysts. Attempts to obtain strains in this way with *S. aureus*, *S. albus* and *Sarcina* were unsuccessful owing to the overgrowth of the cocci by *B. coli* (A^1).

The effects of the floras of strains described in this section (pp. 34, 35) on encystation of *E. histolytica* are briefly tabulated in Table 6.

Table 6. *Effect of bacteria on encystation of* E. histolytica

No.	Strain	Composition of bacterial flora	Encystation
		(i) *K* series	
1	$K+A^1$	A^1	–
2	*Kf*	A^5+N^2	–
3	*KC*	A^5+N^2	–
4	*KA*	A^5+N^2	–
5	$KA+A^4$	A^1+A^4	–
6	$KA+A^3$	A^1+A^3	–
7	*KAP*	A^1 + *B. proteus*	–
8	*KAE*	A^1 + *Enterococcus*	–
9	*KAStr*	A^1 + *Streptococcus*	–
10	KAN^4	A^1+N^4	–
11	KAN^2N^4	$A^1+N^2+N^4$	–
12	*KP*	*B. proteus* + anaerobes*	++
13	*Kp*	*B. prodigiosus* + anaerobes*	++
14	*KSA*	*S. aureus* + A^5 + anaerobes*	±
15	*KN*	Anaerobes* only	±
16	$K+K$	Total flora of *K* 28*c* strain	+++
		(ii) *P* series	
17	$P+A^1$	A^1	–
18	$P+p$	*B. proteus*	–
19	$P+A^3$	A^3	–
20	*PAE*	A^1 + *Enterococcus*	–
21	$P+K$	Total flora of *K* 28*c* strain	++

* The unidentified anaerobes are the spore-bearing organisms from *K* 28*c* flora (N^2, N^4, N^5, N^7).

Inspection of this table will make it clear that the conclusions drawn previously (p. 31) require considerable modification. It appears that *B. coli* is not an essential component of the flora of *K* 28*c*, since the amoebae can undergo their complete development in its absence and can be replaced by other faecal (*B. proteus*) or non-faecal (*B. prodigiosus*) organisms. It is also evident that various bacteria, when in pure culture with the amoebae (A^1, A^3 and *B. proteus*) or in combination with other organisms (A^4, A^5, N^2, N^4, *Enterococcus* and *Streptococcus*), although able to maintain the life of the amoebae indefinitely, were unable to induce encystation.

Series 2

(i) *A strain with four bacteria—KA3N*

As stated above, this strain was prepared by hatching flavine-treated cysts of *K* 28*c* in the presence of *B. coli* (A^1). It grew freely in all the media favourable to *K* 28*c*, especially in Ehs medium, and encysted on the addition of rice starch in the usual way. Cultures lived in the starch-free medium for a week at least and in those containing starch for 10 days or even longer.

Cysts were generally formed in greatest abundance on the 2nd or 3rd day of incubation (slightly later than in *K* 28*c* itself) and were normal in every way. They regularly survived for 2 months or more at 2° C. and produced excellent cultures again on hatching without change of flora. The only constant differences between *KA3N* and *K* 28*c* were that the former gave cleaner cultures, with less turbidity and sediment, while the latter usually produced rather larger crops of cysts under identical conditions. The richness of the amoebic population in the cultures was approximately the same, and hatching was equally good.

From the conclusions drawn in the preceding section it follows that the factor or factors inducing encystation of *E. histolytica* must be associated with the anaerobic bacteria. The anaerobic flora of this strain was therefore analysed further. It was found that, in addition to the anaerobes isolated previously (N^2 and N^4), a third species—which, as has been already explained (p. 25), had been confused with N^4)—was also present. It was named N^5.

(ii) *The anaerobe N^5 and various strains containing it*

This species was isolated by plating out the flora of *KA3N* on blood agar and incubating the plates under anaerobic conditions in a hydrogen jar. After anaerobic incubation on blood agar for 2–3 days, the pure strain grew as very small, flat, colourless, irregular colonies. It also grew readily in pure culture on serum agar and Ehs media, as short clostridia and short coccal chains. Only poor growth was observed on peptone agar. The spores remained viable for 4½ years and the culture, when added to $K+A^1$, continued to promote encystation of *E. histolytica*.

N^5 grew and sporulated freely in pure mixed culture with aerobes, and stock cultures of N^5+A^1 and N^5+A^4 were maintained in Ehs medium. This species of anaerobe in pure culture (incubated anaerobically) or in association with A^4 (incubated aerobically) fermented, with the production of acid

and gas, laevulose, dextrose and maltose. It did not ferment galactose, lactose, mannitol, dulcitol, saccharose, raffinose or salicin.

The effect of the presence of N^5 in cultures of *E. histolytica* was studied by introducing this anaerobe into various strains. It was first added to $K+A^1$, many lines of which, comprising the amoebae and only two bacteria—A^1 and N^5—were synthesized (e.g. *KAN*, see Table 7). They all grew easily in HSre + S and Ehs media and when, after growth for a few subcultures in Ehs medium, the amoebae were transferred to this medium with rice-starch, mature cysts were formed in 3 days. This strain (*KAN*) formed fewer cysts than *K 28c* or *KA3N*, but many ripe cysts were always obtained, and these remained viable for periods up to 88 days when stored at a temperature of 2° C.

Strains of this composition could therefore be maintained indefinitely, in exactly the same way as the parent strains *KA3N* and *K 28c*, although the complex mixture of aerobes and anaerobes of the last-named strains had been replaced by two pure species of bacteria, A^1 and N^5.

In order to test the effect of N^5 when accompanied by different other bacteria, the following strains were prepared: KN^5A^4 (amoebae + A^4 + N^5), KN^5A^2 (amoebae + A^2 + N^5), *K2N* (amoebae + A^1 + N^5 + N^7) and $KA^1N^4N^5$ (amoebae + A^1 + N^4 + N^5). All these strains grew readily and produced cysts in the usual manner after incubation for 2–3 days. The presence of the aerobes A^2 and A^4 and of the anaerobes N^4 and N^7, in addition to A^1 and N^5, did not increase the size of the crops of cysts, nor did they have a harmful effect.

Three other synthetic strains, *KNSt* (amoebae + A^4 + N^5 + *S. aureus*), $KA^1N^2N^5$ (amoebae + A^1 + N^2 + N^5) and KNA^3A^1 (amoebae + A^1 + A^3 + N^5), only formed very few cysts in Ehs + S medium, although amoebae of the two last-named strains grew excellently in Ehs medium with and without rice-starch. The first-named strain, *KNSt*, grew easily in media with rice-starch but very poorly without starch. From these results it is evident that three bacteria—the aerobes *S. aureus* and A^3 and the anaerobe N^2—were detrimental to the production of cysts, but they did not prevent the growth of amoebae in suitable media.

A further strain, *K3N* (amoebae + A^1 + N^2 + N^4 + N^5), was synthesized with three species of anaerobes, and was therefore equivalent to *KA3N*. The amoebae grew well in all the usual media with rice-starch and in Ehs without starch. Good crops of cysts were obtained after incubation for 2 days and the strain was propagated by hatching the cysts. These remained viable when kept for periods up to 45 days at 2° C. The properties of this strain were identical with those of the original *KA3N*.

In order to confirm the results obtained with the strains of simian origin (derived from *K 28c*), a second series of experiments with N^5 were carried out, using the same bacteria but with a human strain of *E. histolytica* (*PC*). As stated above (p. 35), a pure mixed strain growing only with A^1 ($P+A^1$) was prepared and many lines (e.g. *PAN*, see Table 7), composed only of amoebae, A^1 and N^5, were synthesized by adding N^5 to $P+A^1$. Although $P+A^1$ never formed cysts, the strain *PAN* and other identical lines gave good crops of cysts when the amoebae were transferred from Ehs to Ehs + S media. Cysts were obtained in 2 days and in slightly better crops than in *KAN*. Cysts of strain *PAN* were viable for 39 days (stored at 2° C.), and this strain was maintained in the same manner as any other cyst-forming strain of *E. histolytica*. Strain *P2N* (amoebae + A^1 + N^5 + N^7) possessed similar properties to *PAN*.

By treating *PAN* cysts with acriflavine as described above (p. 31), A^1 was killed leaving the cysts accompanied only by spores of N^5. These cysts were hatched by adding a pure culture of an aerobic bacterium, and in this way two new strains were prepared, *PNSt* (amoebae + N^5 + *S. aureus*) and PNA^3 (amoebae + A^3 + N^5). The amoebae of *PNSt* grew well in the usual media when supplemented with rice-starch, but only poor growth was obtained with Ehs medium. When tested for cyst-producing properties in the usual manner, very few cysts were obtained and these were not viable; as in the case of strain *KNSt*, the presence of *S. aureus* was detrimental to encystation.

The 'sterile' flavine-treated cysts of *PAN* were hatched by incubation in Ehs + S medium to which 2 drops of 10% thioglycollic acid had been added. This strain PN^5 (amoebae + N^5) grew in HShs + S and Ehs + S media but would not grow without rice-starch. The growth of amoebae was poor but improved on addition of dead A^1 bacteria or, still better, of dead A^3 (both bacteria were killed by boiling). A few cysts were formed but these were not viable.

PNA^3, prepared as described above, formed viable cysts in the usual manner after 2 days' incubation, the size of the crop being slightly less than in *PAN*. This strain was most valuable since N^5 never sporulated in the cultures; hence by treating the cysts of PNA^3 with acriflavine both A^3 and N^5 were killed, leaving cysts of *E. histolytica* which were sterile, i.e. unaccompanied by any other organisms. The flavine-treated cysts were hatched by incubation with various aerobes, A^1, A^3 and *B. proteus*. In this way, many pure mixed strains were prepared. The properties of these strains are described above (p. 35).

The cyst-producing properties of all strains containing living N^5 are briefly summarized in Table 7.

As living N^5 were found to promote cyst-formation, experiments were made to test the effect of dead N^5 and filtrates of pure N^5 cultures.

Table 7. *Effect of bacteria on encystation of strains of* E. histolytica *containing the anaerobe* N^5

No.	Strain	Composition of bacterial flora	Encystation
		(i) *K* series	
1	*KAN*	A^1+N^5	++
2	KN^5A^4	A^4+N^5	++
3	KN^5A^3	$A^1+A^3+N^5$	++
4	KNA^3A^1	$A^1+A^3+N^5$	−
5	*KNSt*	$A^4+N^5+S.$ *aureus*	±
6	*K2N*	$A^1+N^5+N^7$	++
7	$KA^1N^4N^5$	$A^1+N^4+N^5$	++
8	$KA^1N^2N^5$	$A^1+N^2+N^5$	±
9	*K3N* (=*KA3N*)	$A^1+N^2+N^4+N^5$	+++
		(ii) *P* series	
10	PN^5	N^5	±
11	*PAN*	A^1+N^5	++
12	PNA^3	A^3+N^5	++
13	*PNSt*	*S. aureus*+N^5	−
14	*P2N*	$A^1+N^5+N^7$	++

The liquid portion of a $K+A^1$ culture in Ehs medium was removed and replaced by a suspension of an autoclaved pure culture of N^5. After incubation in this medium for 3 days, a few mature cysts were seen in clumps of dead N^5. These cysts were viable and hatched after 45 days' storage at a low temperature. The filtrates of N^5 were prepared by filtering the liquid part of pure cultures of N^5 in Ehs medium through gradocol membranes (pore size varied from 0·68 to 0·71 μ). The liquid so obtained was added to egg slopes instead of the diluted horse serum. When grown in this medium amoebae of $K+A^1$ formed a few cysts after 3 days. These cysts hatched after being kept for 7 days at room temperature.

In spite of this success in inducing cyst-formation with $K+A^1$, all attempts to repeat these experiments with $P+A^1$ failed.

From the experiments with N^5 it is seen that, among all the bacterial components of strain *K* 28*c*, this anaerobe is the only organism capable of providing the essential factors for inducing cyst-formation in *E. histolytica*. Suitable conditions for the growth of the amoebae with N^5 may be provided by various species of aerobic bacteria or by adding thioglycollic acid to the medium, but these two methods are not equally good for inducing cyst-formation in the presence of N^5. If the chemical method is used, the amoebae growing in pure culture solely with N^5 will encyst only in small numbers and the cysts are not viable. However, cyst-formation is improved when an aerobe is also present, though not all aerobes are equally suitable for this purpose. Thus, A^1, A^2 and A^4 improve encystation, whereas *S. aureus* reduces or even inhibits cyst-formation. A^3 occupies an intermediate position, since cysts were easily formed by strain PNA^3 (with a flora of A^3 and N^5), but the addition of A^3 to strain *KAN* inhibited cyst-production. It is evident that anaerobiosis is not the only condition supplied by an aerobe, but the increase of the cyst crop may also be due to the nutrition provided by the vegetative bacterial cell.

It must also be pointed out that the cyst-producing capacity of any strain was not correlated with the ease of growth of the amoebae in media containing rice-starch. For instance, the amoebae of strains growing with *S. aureus* as the accompanying aerobe flourished in such media, but this species of bacteria prevented the formation of cysts, even when N^5 was the only other bacterial organism present. The addition of other anaerobic bacteria, N^4 and N^7, to strains of *E. histolytica* containing a favourable aerobe plus N^5 did not influence encystation, though otherwise these species appear to be indifferent. On the other hand, N^2 proved to be detrimental to cyst-production.

Although it may be deduced from the results of experiments with pure cultures that the various constituents of the *KA3N* or *K* 28*c* floras are either harmful or beneficial to cyst-formation, these conclusions do not apply to cases when these bacteria form part of a complex mixture. Thus, the harmful effect of N^2 which is manifested in pure cultures is lost when this anaerobe is grown together with N^4 and N^5, for the resulting strain (*KA3N*) produces larger crops of cysts than any strain consisting solely of a favourable aerobe and N^5. Similarly, strain *K* 28*c*, although it contains at least two organisms (A^3 and N^2) which have been shown to reduce or inhibit cyst-production when examined separately, produced larger crops or cysts than either *KA3N* or any simpler synthetic strain.

III. Summary and conclusions

1. It has previously been shown [by Dobell] that, in cultures of strain *K* 28*c*, *Entamoeba histolytica* could be maintained indefinitely and made to pass through all the stages of its life history.

2. It was also demonstrated that all non-encysting strains of *E. histolytica* could be induced to encyst *in vitro* by substituting their concomitant bacterial flora by that of strain *K* 28*c*.

3. In order to ascertain what part each constituent of the bacterial flora of *K* 28*c* played in the life of *E. histolytica*, a detailed analysis of this flora was carried out. It was found to comprise eight aerobic organisms (mostly of the *B. coli* group) and six anaerobes.

4. The properties of individual species and

varieties of bacteria were studied by adding them to cultures of the amoebae or by hatching the cysts of *E. histolytica* in media previously seeded with the bacteria to be tested.

5. With the same end in view two methods were devised for producing simplified strains of *E. histolytica* associated with from one to four species of bacteria only. By treating the cysts with acriflavine, the mixed flora could be freed of the aerobic organisms, leaving only the spores of the anaerobes. The spore-bearing anaerobes, in their turn, could be eliminated by treatment of the mixture with gentian violet. By using these methods it was possible to produce cultures of 'synthetic' strains of *E. histolytica* accompanied by any desired combination of bacteria, whose properties it was desired to investigate.

6. The results of the earlier experiments suggested that the essential constituent of the flora of *K* 28*c* was *B. coli*, all varieties of which appeared to promote growth, induce encystation and excystation, and generally favoured the completion of the life-cycle of the amoebae *in vitro*, whereas the anaerobic bacteria present in the strain appeared to be inactive as regards encystation.

7. However, later experiments have demonstrated conclusively that, although the aerobic bacteria individually induced the cysts of *E. histolytica* to hatch and supported the growth of the amoebae indefinitely, only one species—the anaerobe N^5—was capable of promoting encystation. As regards the aerobic bacteria, their chief role is to provide the anaerobic conditions necessary for the growth of the amoebae with N^5 in culture.

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EXPERIMENTAL PRODUCTION OF PURE MIXED STRAINS OF *ENTAMOEBA HISTOLYTICA*

By R. A. NEAL, Ph.D., *Wellcome Laboratories of Tropical Medicine, London*

I. INTRODUCTION

In this issue of *Parasitology* (p. 16) there appears a paper by the late Dr Dobell, in which a method for preparing cultures of pure mixed strains of *Entamoeba histolytica* is described. As that paper was completed by me on the basis of Dobell's note-books, the publication of his posthumous work was delayed until confirmatory experiments had been performed (see also Dobell, *loc. cit.* p. 34). In the present paper, these and some related experiments are described.

II. MATERIAL AND METHODS

Although in the experiments described below several different strains are used, the most important of these, from which the others were derived, is strain *RD*. This strain was isolated on 5. ix. 50 from the faeces of a rat which had been experimentally infected with a human strain of *E. histolytica* (strain *MDS*). At the time of isolation of strain *RD*, the rat had remained infected for 15 months (see Neal, 1951).

The amoebae of this strain, therefore, are ultimately of human origin, but the bacterial flora is probably a mixture of the original flora and of murine bacteria. The flora of strain *RD* has not been systematically analysed, but so far two different aerobes and two anaerobes have been recognized.

After isolation *in vitro*, this strain was observed to form good crops of cysts when treated in the manner described by Dobell & Laidlaw (1926). Cysts were formed in Ehs + S* medium, after 2 days' incubation at 37° C., and they remained viable for periods up to 3 months when stored in the refrigerator (4° C.).

Other strains of *E. histolytica* used were strain *XK* and Dobell's *K* 28*c*. The former strain was similar to *RD* in that it was isolated *in vitro* from a rat experimentally infected with a human strain of *E. histolytica* (strain *M*; see Neal, *loc. cit.*). The history of *K* 28*c* is described in the preceding paper (Dobell, *loc. cit.*).

The species of aerobic bacteria used in my experiments were *Chromobacterium prodigiosum* (NCTC strain No. 1337) and *Bacterium coli* (strain A^1). The strains of *B. coli* and *E. histolytica* (*K* 28*c*) were given to me by Dr Dobell shortly before his death. The usual bacteriological technique was followed for determining the presence of various bacteria.

For eliminating the anaerobic bacteria one sample of gentian violet, manufactured by Grübler, was used.

* The symbols employed for the media are those used by Dobell (loc. cit.).

III. EXPERIMENTS WITH RAO'S METHOD OF PREPARING PURE MIXED STRAINS OF *E. HISTOLYTICA*

Recently, Rao (1951) has described a method for preparing pure mixed strains of *E. histolytica*, which consists of treating the cysts successively with mercuric chloride (0·002 % for 45 min.), potassium permanganate (0·02 % for 15 min.) and acriflavine (0·02 % overnight), all stages of treatment being carried out at room temperature. After washing, the cysts were hatched in a medium consisting of liver infusion agar slants covered with diluted horse-serum to which rice-starch was added. This medium had been seeded with either *Bacterium coli* or *C. prodigiosum* and incubated for 24 hr. (at 37° or 25° C. respectively) prior to inoculation with the treated *E. histolytica* cysts. The author claims to have succeeded in establishing *E. histolytica* in pure culture with *B. coli* and *C. prodigiosum* in two experiments. In one, cysts were obtained from culture, in the other from human faeces.

As this method appeared to be much quicker and easier than that described by Dobell, I have tried it out in my experiments. In all the experiments except two, Rao's method was followed exactly, except that the medium employed for hatching the cysts was HSre + S instead of liver infusion agar-horse serum medium. In two experiments the liver infusion agar medium was used as described by Rao, and these are noted below.

The first experiment was carried out using strain *RD*. After treating the cysts with the chemicals in the manner described above, they were inoculated into three tubes of HSre + S medium, one seeded with *C. prodigiosum*, one with *B. coli*, and one—which served as a control—with no added bacteria. After 2 days' incubation at 37° C. amoebae were observed growing in the tube with *C. prodigiosum* (this strain was named *Dpr*) and after 3 days' incubation in the tube with *B. coli* (strain DA^1). The control tube

showed no growth of bacteria after 3 days' incubation. After incubation for 24 hr. the two strains showed a pure growth of the aerobes, *C. prodigiosum* and *B. coli* respectively, but later examinations revealed the presence of many anaerobic bacteria, which included both Gram + and Gram − organisms. These strains of *E. histolytica* were maintained for many weeks and the anaerobic bacteria were always present. Since subsequent experiments showed that *Dpr* and *DA*[1] encysted readily, Rao's method was repeated with cysts from these strains. The treated cysts were again hatched in HSre + S medium with *C. prodigiosum* and *B. coli*. Although the flora of these strains (*Dpr* and *DA*[1]) had already been simplified, the anaerobes were not eliminated by the second treatment, and each strain gave mixtures of aerobic and anaerobic bacteria as in the parent strains *Dpr* and *DA*[1].

One further experiment with these strains was carried out. As described below, a pure strain consisting of amoebae plus *C. prodigiosum* (*D* + *pr*) was prepared from *Dpr* by Dobell's method. To this pure strain, the total bacterial flora of *Dpr* strain was added, thus synthesizing the strain *Dpr*. The synthetic strain was called *DN*. Culture cysts of strain *DN* were treated with Rao's chemicals and hatched in liver infusion agar-horse serum medium with *B. coli* and *C. prodigiosum* as described by Rao. The cysts incubated with *B. coli* hatched and amoebae were observed on the 4th day of incubation, but no cysts were hatched in the tube with *C. prodigiosum*. In both tubes, however, anaerobic bacteria, as well as the added aerobes, were detected when examined on the 5th day of incubation. The control tube incubated aerobically was sterile. The anaerobes were always present on continued subculture.

Other similar experiments were carried out with strain *K* 28*c*, using both types of medium for hatching the cysts, and with *XK* using HSre + S medium only, and both strains gave similar results, for although all non-sporulating organisms were eliminated, spore-forming anaerobes were always present.

IV. PREPARATION OF A PURE MIXED STRAIN OF *E. HISTOLYTICA* BY DOBELL'S METHOD

The method devised by Dobell is divisible into two stages. In the first stage the mixture of aerobes are killed and replaced by one species of aerobic bacteria, and in the second stage the anaerobes are eliminated by the use of gentian violet in the manner described below.

In this experiment the aerobic organisms present in the bacterial flora of *RD* were killed by the method devised by Rao (1951), leaving the cysts of *E. histolytica* accompanied by the spores of the anaerobic bacteria.* The cysts—and the bacterial spores—were then hatched in HSre + S medium in the presence of a pure culture of *C. prodigiosum*. The amoebae together with the concomitant bacteria were subcultured daily in the following manner. A tube of HSre + S medium was prepared with only 1 ml. of liquid overlay (re); this was inoculated with 2 drops of a broth culture of *C. prodigiosum* and incubated overnight to obtain a good growth of the aerobe. Just before inoculating the amoebae, about 0·5 ml. of 0·2 % gentian violet was added to the 1 ml. of liquid overlay of the medium (re). The dye was carefully mixed with the liquid in order to avoid any undue oxygenation. The amoebae were then carefully inoculated at the bottom of the slope. The numbers of amoebae present, when subcultured daily in this manner, decreased but a few managed to survive. The amoebae frequently ingested the dead bacteria stained purple by the dye. After 33 daily subcultures the bacterial flora was found to be a pure culture of *C. prodigiosum*. Subcultures of two lines of the pure strain *D* + *pr* were kindly examined by Brigadier J. S. K. Boyd, F.R.S., who confirmed the presence of only one species of bacteria.

Two precautions must be noted. First, the serum slope must be freshly prepared, so as to ensure that the slope has not shrunk at the bottom and detached itself from the wall of the tube, thereby allowing the amoebae to fall into the space thus formed. If this occurs, then within 24 hr. the serum slope will adsorb the dye almost completely from the small volume of liquid filling the crack, leaving the liquid completely colourless and enabling the amoebae and—more important—all the types of bacteria present to develop unhindered. Secondly, it is essential to subculture daily, since—apart from local adsorption of gentian violet—the whole length of the slope will slowly adsorb the dye so that within 3 days of incubation the slope will be deeply stained and the concentration of gentian violet in the liquid will not be sufficient to kill or even check the multiplication of anaerobic bacteria. If too high a concentration of gentian violet is used, the dye will kill all aerobic (Gram −) bacteria in addition to the anaerobes and the amoebae will be unable to survive.

The strain *D* + *pr* was maintained in HSre + S medium, and survived regularly for seven days and often up to 15 days. It grew in all the usual media supplemented by rice-starch, but amoebae were never abundant. Usually only 1–5 amoebae were seen in each microscopic field (16 mm. objective and 10 × ocular). In Ehs medium it grew very poorly and was difficult to propagate. It never produced cysts on transferring to Ehs + S medium.

* This method was used because the experiments with Rao's technique had just been completed, providing a suitable strain for this work. A similar strain could have been obtained by Dobell's acriflavine method.

V. DISCUSSION

The experiments described above confirm and provide further details of Dobell's method (1952) for purifying strains of *E. histolytica*, which is based on Churchman's (1912, 1913) and Churchman & Michael's (1912) demonstration that Gram + bacteria are more sensitive to gentian violet than Gram − organisms. The same effect was noted by Howitt (1926), who also observed that *E. gingivalis* was able to withstand strong concentrations of this dye. Dobell's method is easy to perform for those workers experienced in handling *E. histolytica* cultures, although several attempts with a particular strain may be necessary. Success is dependent upon maintaining an optimum concentration of gentian violet, which will just allow the amoebae to grow and yet inhibit the growth and prevent sporulation of the Gram + anaerobes. That the concentration of the dye is sufficient to inhibit the growth of spore-forming Gram − organisms as well was shown by Dobell (*loc. cit.*) in the case of N^4.

The results of experiments using Rao's (1951) method prove that his technique did not sterilize *E. histolytica* cysts, but merely killed all non-sporing bacteria. The spores of the anaerobes, together with the cysts of *E. histolytica*, hatched when incubated in the appropriate medium in the presence of an aerobic species of bacterium, and a further treatment with gentian violet was required to remove the anaerobes. In fact, with the strains I have used, Rao's method gave a result similar to that obtained with the acriflavine technique described by Dobell (1952).

This criticism of Rao's work may be applied equally to other methods for sterilizing *E. histolytica* cysts by means of various chemicals, which have been described in the literature (for a review of these papers see Rao, 1951; also Ishino, 1949 *a*, *b*). A possible exception is the method of Balamuth & Wieboldt (1951), where cysts were obtained from a strain with a bacterial flora consisting only of non-sporing aerobes. In the light of Dobell's work, where he showed that the encystment factor was provided by only a spore-bearing anaerobe, Balamuth & Wieboldt's work is particularly interesting, since their strain does not apparently contain any such organisms. In the absence of any spores, treatment of cysts by a suitable method would easily sterilize the latter and provide all the conditions for a pure culture of *E. histolytica*.

All strains of *E. histolytica* examined by me contained spore-bearing anaerobic organisms—in addition to aerobic bacteria. In view of the fact that such spore-bearing bacteria are normal inhabitants of the large intestine of man, the chances of isolating a strain of *E. histolytica in vitro* in the usual way, without these organisms forming part of the bacterial flora, are so small as to be practically negligible.

I am still maintaining Dobell's strain *K* 28*c* and will be pleased to supply cysts to anyone wishing to repeat Dobell's work. A strain of *E. histolytica* growing only with *B. coli*—$P + A^1$—prepared by Dobell, is being maintained by Dr J. D. Fulton at the National Institute of Medical Research, London.

VI. SUMMARY

1. Dobell's observations on the production of pure mixed strains of *Entamoeba histolytica* have been repeated and confirmed.

2. It has been demonstrated that the method devised by Rao did not sterilize the cysts of *E. histolytica* but merely killed all non-sporing organisms, while the spores of all spore-forming bacteria were unaffected.

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THE FOOD HABITS OF *ENTAMOEBA HISTOLYTICA* IN ITS COMMENSAL PHASE

By CECIL A. HOARE, F.R.S., *Wellcome Laboratories of Tropical Medicine, London*

(With 1 Figure in the Text)

INTRODUCTION

In a number of previous publications (Hoare, 1947, 1949*a*, *b*, 1950) I have discussed the host-parasite relations in human amoebiasis, which were summarized as follows (Hoare, 1950, p. 240): 'Although *E. histolytica* is a pathogenic parasite, capable of invading the tissues of its host with the production of lesions and clinical symptoms of disease, in most cases it does not manifest any evidence of virulence, for in up to 90% of persons harbouring this parasite the infection is symptomless and the host is a carrier. In most carriers the amoebae live in the lumen of the gut as harmless commensals and represent the only stage capable of producing cysts, which serve to propagate the infection to new hosts. But under certain conditions the lumen-dwelling amoebae may reveal their virulence and invade the intestinal wall, giving rise to the typical symptoms of amoebiasis.' This conception has been steadily gaining support since it was first fully enunciated by Kuenen & Swellengrebel (1913), and is now more or less generally accepted in continental Europe. However, it has not met with the same success in Great Britain and in America, where many clinicians and parasitologists still regard *E. histolytica* as an obligatory tissue-parasite which invariably invades the intestinal wall, producing the characteristic gross or minute lesions and feeding on erythrocytes. They believe that lesions—however minute—are also present in all carriers, thus denying the very existence of *healthy* symptomless cases. Among the early critics of a commensal mode of life in *E. histolytica* was Dobell (1919), but he subsequently changed his views; its strongest opponents at present are Faust (1941) and Craig (1944).

The existence of a commensal phase in the life history of *E. histolytica* is based on the following considerations: (1) In natural and/or experimental infections of monkeys and rats, the amoebae usually live in the lumen of the gut without damaging its walls (Dobell, 1928, 1931; Neal, 1948, 1951). (2) In stools passed by symptomless human carriers, there may appear—after administration of purgatives—enormous numbers of non-haematophagous amoebae (Reichenow, 1913). (3) Direct examination of the intestinal wall in amoebic carriers—by sigmoidoscopy (Morton, Neal & Sage, 1951) and *post mortem* (Acton, 1933; Faust, 1941)—reveals an intact mucous membrane. (4) Strong circumstantial evidence was provided by the benzidine test for occult blood: when applied to the stools of amoebic carriers it failed to reveal any traces of blood, the presence of which would have been expected if the amoebae resided in the tissues (Andrews & Atchley, 1932). (5) The amoebae feed on bacteria, both *in vivo* (in monkeys, rats and human carriers: vide supra, (1) and (2)) and *in vitro* (in culture).

Since the indirect indications of a commensal existence of *E. histolytica* have been more fully discussed elsewhere (Hoare, 1950), in the present paper I propose to deal only with the food habits of this amoeba, which provide direct evidence of its mode of life in man. That *E. histolytica* can subsist on bacteria *in vitro* has been established beyond all doubt since the discovery of suitable culture media in 1925. Previously, a number of investigators—especially Kuenen & Swellengrebel (1913)—have pointed out that in cases of chronic amoebiasis and in carriers the amoebae live in the lumen of the human bowel, where they feed on micro-organisms and faecal debris. These observations were subsequently confirmed and extended by other workers, some of whom have illustrated their papers with figures showing the amoebae with ingested bacteria. As I intend to give a detailed review of these works in another publication, I shall not dwell on them here.

There is still a strong bias against the acceptance of a commensal phase in *E. histolytica*, which these findings imply. Over 30 years ago Dobell was among the foremost opponents of this view, and in his famous monograph (Dobell, 1919) he wrote: 'On purely *a priori* grounds the hypothesis is highly improbable. It is unlikely that an amoeba which is generally dependent upon living tissues for its nourishment should at times completely change its habits and become a feeder on bacteria.' However, Dobell's subsequent investigations on the behaviour of *E. histolytica* in cultures and in monkeys brought about a radical change in his views, for he states (Dobell, 1928) that 'in their natural host [macaque monkey] the amoebae... ingest bacteria. They neither invade nor feed upon the tissue of the large intestine,' and later (Dobell, 1931) adds: 'It appears

almost certain now that *E. h. hominis** sometimes lives in men as a harmless commensal—just as *E. h. macacorum** typically does in its natural hosts.' Though the number of observers who have described and depicted the ingestion of bacteria by *E. histolytica* in human infections is considerable, many workers still adhere to Dobell's earlier views and stubbornly refuse to admit a commensal mode of existence in the 'dysentery amoeba'.

MATERIAL AND METHODS

Having been interested in this phenomenon for some years, I have taken every opportunity to examine the stools from symptomless carriers and from cases of chronic amoebiasis. As the result of these studies I was able to satisfy myself—and demonstrate to others (Hoare, 1949*a*)—that in such persons a fair proportion of the amoebae contained food-vacuoles enclosing bacteria, which had obviously been ingested from the faecal contents in the lumen of the gut. The available material consisted, on the one hand, of old stained faecal preparations from suitable cases placed at my disposal by the late Dr C. M. Wenyon, F.R.S., and, on the other hand, of recent faecal preparations stained by myself; these were kindly provided by Dr J. P. Caplan and Air Vice-Marshal T. C. Morton, to whom my thanks are due. The preparations were all made from fresh faeces; in the case of the older slides this was evident from the normal appearance of the amoebae, while the recent slides were actually made immediately the stools had been passed. The preparations were stained with Heidenhain's iron haematoxylin or Dobell's tungstic haematoxylin.

The material at my disposal comprised preparations from four cases of chronic infection, two convalescent carriers and one contact carrier, the history of which is as follows:

Nos. 1–4. Cases (A, B, C, D) of chronic amoebiasis from Panama, stained faecal preparations of which were prepared in 1913 and sent to Dr Wenyon by the late Dr W. M. James, who was then engaged in a special study of *E. histolytica* in chronic and carrier cases. The slides are still in a perfect state of preservation.

No. 5. Convalescent carrier (Case Healy) from Egypt, whose stained faecal smears were prepared by Dr Wenyon in 1916. According to Wenyon & O'Connor (1918), and Dr Wenyon's verbal communication, this patient had suffered on and off for 5 years from relapsing amoebic dysentery, acute attacks of which alternated with latent periods, when unformed stools containing large numbers of amoebae and cysts were passed. The available faecal preparations—still in excellent condition—were made when the patient was in the carrier state.

No. 6. Case (S) of chronic amoebiasis from a London hospital. According to Dr J. P. Caplan, the patient had been suffering from chronic relapsing dysentery for several years (1943–8). The disease did not respond to drugs till 1948, when the last course of anti-amoebic treatment was given and the patient ceased to relapse. The faecal preparations were made when the patient was in effect a convalescent carrier.

No. 7. Contact carrier: the patient, who was in the R.A.F., was invalided to England from the Far East in 1948 suffering from a form of neurosis. Routine examinations of his stools revealed the presence of *E. histolytica* cysts, but there were no symptoms of intestinal disorder. Smears made from a fresh formed stool were supplied through the courtesy of Air Vice-Marshal T. C. Morton.

Before turning to my own observations, it is necessary to consider the objections raised by opponents of a commensal phase in *E. histolytica*. The majority of them base their criticism merely on the assumption that the amoebae always invade the intestinal wall with the production of lesions, and dismiss without any explanation the reported presence of bacteria in the amoebae; they thus commit the logical error of begging the question. But one or two authors have discussed these findings at some length. Thus Dobell (1919) believed that bacteria are not ingested by healthy amoebae, but these micro-organisms merely invade degenerating or dead individuals found in stale stools; such amoebae can be recognized by abnormal nuclear structure. He also observes that the bacteria within the amoebae may be there as parasites, a possibility which is also discussed by Epstein (1941), who adds that in such cases the bacteria are not enclosed in food-vacuoles. Dobell further notes that rod-shaped or granular chromatoid bodies, sometimes seen in precystic amoebae, may be mistaken for bacteria; and he finally points out that there must be proof that the amoebae with ingested bacteria are *E. histolytica* and not *E. coli*.

All these, and other possible sources of fallacy in the interpretation of the food habits of *E. histolytica*, have been taken into consideration in the present study. It has already been noted above that all the preparations were made from fresh stools. The amoebae examined for food-inclusions were normal healthy individuals—some of them fixed in the process of locomotion with extended pseudopodia (Fig. 1, *d*, *f*), while the nucleus of none of them showed any signs of degeneration but was structurally typical of *E. histolytica*. In addition to nuclear structure, their specific identity was confirmed by the presence

* The designations *E. histolytica hominis* and *E. h. macacorum*, as used by Dobell, are not intended to denote subspecies but merely indicate strains of human and simian origin respectively.

in the stools of all the cases examined of typical quadrinucleate cysts. The presence of bacterial infection in my preparations is very unlikely; first, because the bacteria found within the amoebae were represented by a variety of forms, all of which were also present in the surrounding faeces; and, secondly, because in many cases they were surrounded by food-vacuoles (see Fig. 1, *b–d*). As regards the possible confusion of small chromatoids with bacteria, this was excluded by listing doubtful cytoplasmic inclusions separately (see Table 1). Finally, there is another source of error, not mentioned by other observers. Since the amoebae in faecal preparations are usually encrusted with bacteria, particular care was taken to exclude the latter and record only those which were undoubtedly inside the body of the amoeba. This could easily be ascertained (1) by careful focusing, (2) by the presence of food-vacuoles enclosing the micro-organisms, and (3) by the unequal degree of differentiation of the bacteria lying inside and outside the amoeba, after staining with haematoxylin.

In examining each preparation I recorded the appearance and cytoplasmic inclusions of the trophic amoebae in the order in which the latter appeared in view, excluding those individuals which showed any abnormality in structure (especially the nucleus). In most cases 100 individuals were inspected, but in two cases (Nos. 2 and 6) with very scanty infections fewer numbers were counted.

RESULTS

The results of these observations are set forth in Table 1, from which it is seen that in chronic infections and in carriers from 30 to 100 % of the amoebae contained bacteria, from 2 to 40 % contained particles of undetermined nature, and from 0 to 45 % were free of any inclusions, while none of the amoebae contained erythrocytes. The number of ingested bacteria in different amoebae varied and was sometimes considerable (e.g. Fig. 1, *c–f*). They were represented chiefly by cocci, diplococci, bacilli and 'bipolar' bacteria, which corresponded to the organisms seen in the surrounding faecal matter. It was evident that the amoebae feed indiscriminately on all kinds of bacteria, since these occurred in their cytoplasm alone or in different combinations. It was not always easy to detect food-vacuoles, but in many cases these—with enclosed bacteria—were clearly visible (Fig. 1, *b–e*).

The accompanying figures—better than any description—convey an idea of the variety of the bacteria seen and of their distribution in the body of the amoebae. For comparison with the amoebae in human infections, I have also depicted amoebae with ingested bacteria found in the intestinal contents of monkeys (Fig. 1, *g*) and rats (Fig. 1, *h*) experimentally infected with human strains of *E. histolytica*. The other figures show ingestion of red blood corpuscles in amoebae from a human case of

Table 1. *Food-inclusions in* Entamoeba histolytica

No.	Cases	Number of amoebae counted	Inclusions (%)			
			Bacteria	Undetermined	R.B.C.	Absent
1	Chronic infection: Case A. Panama, 1913	100	32	40	—	28
2	Chronic infection: Case B. Panama, 1913	50	30	32	—	38
3	Chronic infection: Case C. Panama, 1913	100	64	17	—	19
4	Chronic infection: Case D. Panama, 1913	100	30	33	—	37
5	Convalescent carrier: Case Healy. Egypt, March 1916	100	63	25	—	12
	May 1916	100	63	15	—	22
6	Convalescent carrier: Case S. England, February 1948	20	100	—	—	—
7	Contact carrier: Case R.A.F. England, November 1948	100	53	2	—	45

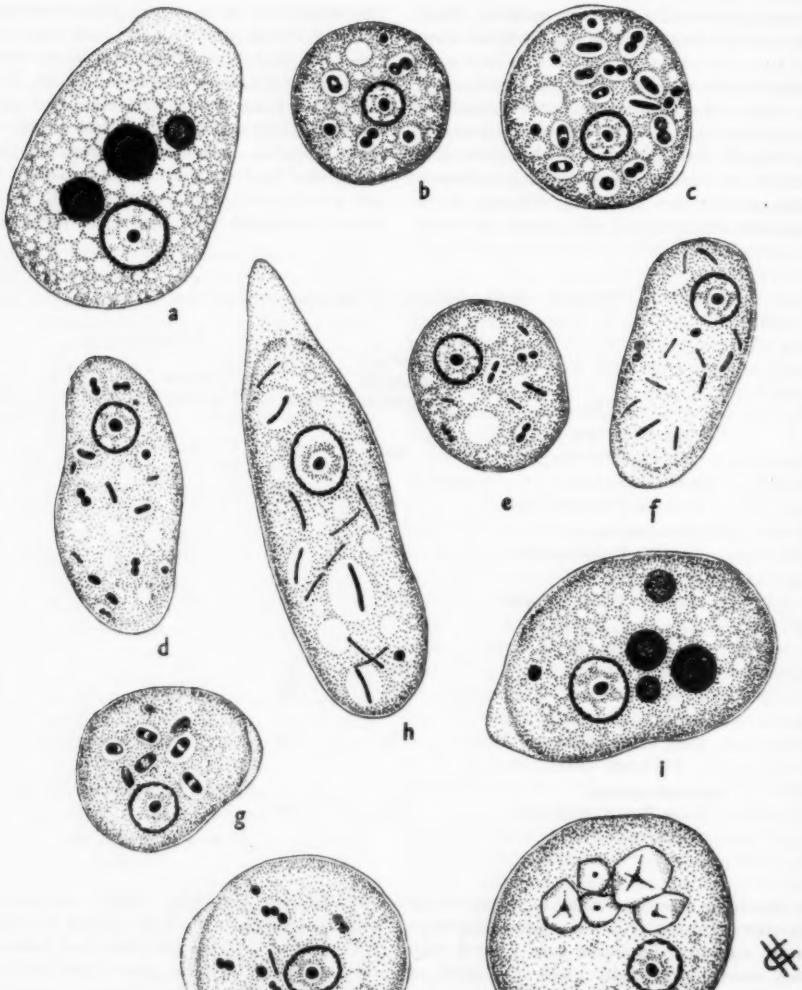

Fig. 1. Food habits of *Entamoeba histolytica* (× 2000). *a*, amoeba with ingested erythrocytes, from a case of acute amoebic dysentery; *b*, amoeba with ingested bacteria, from a contact carrier (no. 7); *c*, amoeba with ingested bacteria, from a convalescent carrier (no. 6); *d–f*, amoebae with ingested bacteria, from cases of chronic amoebiasis (nos. 1, 3, 4); *g*, amoeba with ingested bacteria, from monkey infected with human strain; *h*, amoeba with ingested bacteria, from rat infected with human strain; *i*, amoeba with ingested erythrocytes, from rat infected with human strain; *j*, amoeba with ingested bacteria, from culture; *k*, amoeba with ingested starch granules, from culture. (The numbers in brackets refer to the cases listed in Table 1.)

acute dysentery (Fig. 1, *a*) and from an experimentally infected rat (Fig. 1, *i*), as well as the food-inclusions of amoebae in cultures (Fig. 1, *j*, *k*).

Under the heading 'undetermined inclusions' (see Table 1) I have listed particles the nature of which was doubtful; these might have been bacteria or faecal debris in the process of digestion, or even small chromatoid bodies, which sometimes occur in precystic amoebae. As regards the amoebae with a clear cytoplasm, they were either individuals feeding saprozoically or precystic amoebae, which had ceased to ingest food. The majority of the amoebae seen were of medium size, being somewhat larger than the precystic forms but smaller than the haematophagous forms (Fig. 1, *a*) found in dysenteric stools; they thus fully correspond to the so-called *minuta* stage of *E. histolytica*.

CONCLUSIONS

These observations on the food habits of *E. histolytica* prove beyond doubt that in chronic and carrier cases of amoebiasis the trophic amoebae in man habitually feed on bacteria, as they usually do in cultures, and in monkeys and rats. Since these bacteria are normal constituents of the human intestinal flora, it can be inferred that the natural site of the amoebic infection in such cases is the lumen of the large bowel, in which the amoebae lead a commensal existence, like *E. coli*. However, the food habits of *E. histolytica* in the human host may vary according to the course of the infection, for—unlike its congener—it can invade the intestinal wall and start feeding on erythrocytes and tissue elements. That *E. histolytica* is omnivorous is also evident from the fact that its food habits can be changed at will. Thus, when the commensal forms of the amoebae are transferred from man to a kitten, they may attack its gut wall and feed on red cells, but if the amoebae from the infected kitten are recovered in culture, they revert to their original diet and ingest bacteria.

In the light of these data, Dobell's earlier pronouncement (cf. p. 43 supra) can be paraphrased as follows (Hoare, 1947): 'The position of those who regard *E. histolytica* as an obligatory tissue parasite is untenable even on *a priori* grounds, for it is inconceivable that an organism feeding exclusively on tissue elements and erythrocytes in the human host should completely change its habits in other mammalian hosts and in culture, and become a feeder on bacteria.'

SUMMARY

1. After a general consideration of the host-parasite relations between *E. histolytica* and man, a case is made for the recognition of a commensal phase in the life history of this amoeba.

2. This view is supported by observations on the food habits of *E. histolytica* in chronic cases and carriers of human amoebiasis.

3. From these observations it is evident that from 30 to 100 % of trophic amoebae in such cases have ingested bacteria derived from the contents of the large bowel.

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THE OCCURRENCE IN PAKISTAN OF A HUMAN *ENTAMOEBA* OF THE *POLECKI* TYPE*

By GEORGE LUBINSKY, M.D., *Major, Pakistan Army Medical Corps.*
Central Military Pathological Laboratory, Rawalpindi, Pakistan

(With 9 Figures in the Text)

INTRODUCTION

During a routine examination of intestinal protozoa in a small group of healthy persons in Rawalpindi, Pakistan, I found in one person uninucleate amoebic cysts which, in the preliminary examination of an iodine-stained preparation, I attributed to *Entamoeba histolytica*. However, the examination of a haematoxylin-stained preparation did not confirm this diagnosis, for the cysts in question were found to be morphologically identical with those described by Kessel & Johnstone (1949) in human beings in the U.S.A. and in monkeys (*Macaca mulatta*) imported to the U.S.A. from India. These authors found uninucleate amoebic cysts with a peculiar inclusion mass in their cytoplasm and a nucleus containing a large karyosome. They call their amoeba *Entamoeba polecki*, a name under which Prowazek (1912) described an amoeba found by him in the faeces of pigs and—on one occasion—in a child in Saipan, Ladrone Islands (Mariannes). Very briefly—in 14 lines—he describes the variable structure of the nucleus, which he explains by cyclical changes in it (earlier described by Hartmann in other amoebae). The presence of a peculiar 'sexual process' occurring in stale stools, is also mentioned.

Dobell (1919), in the famous monograph on the amoebae living in man, says about this species: 'There is nothing to show that this "species" is not a mixture of amoebae from pigs and human beings confused with free-living species from stale human stools.' According to Hoare (1940) the unacceptability of the name *E. polecki* has also been noted by a number of other authors. In the same paper Hoare critically examines the species *E. polecki* and concludes that this name has to be rejected as *nomen nudum*. Therefore, in accordance with the International Rules of Zoological Nomenclature, this name cannot be used for any organism. But if it can be proved that the amoeba described by Kessel & Johnstone is a new species, it will have to be renamed (Hoare, *in litteris*). In the present paper the designation 'amoeba of the *polecki* type' will be used to stress the morphological identity of cysts found by me with those described by Kessel & Johnstone in 1949.

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MATERIAL AND METHODS

The cysts of the *polecki* type were found in an apparently healthy man, aged 28, domiciled in Punjab. The anamnesis revealed no contact with pigs or monkeys. Six formed stools and one obtained after the administration of magnesium sulphate were examined in April and May 1950, while four formed stools and two obtained after administration of Epsom salt were examined in October–December 1950. For the examination of formed stools the zinc sulphate flotation method was applied. Smears of both formed and fluid stools were fixed with warm Schaudinn's fluid (with the addition of 5% glacial acetic acid) and stained with Heidenhain's iron haematoxylin or with haemalum.

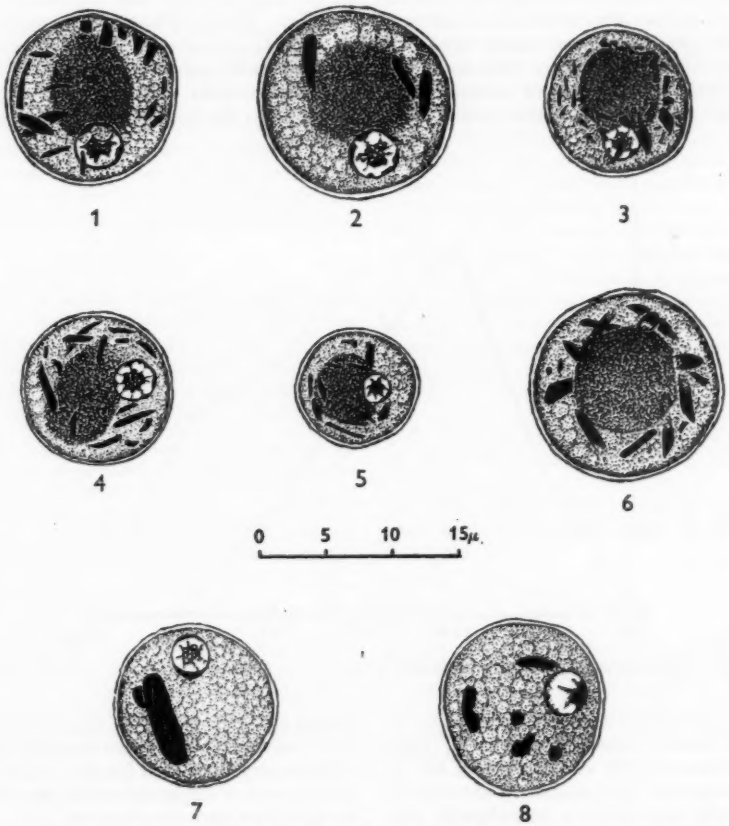
RESULTS

The *polecki* type of cysts were found in the spring in stool samples, which also contained the following protozoa:

1. *Entamoeba coli*.
2. *Endolimax nana*.
3. *Iodamoeba bütschlii*.
4. *Chilomastix mesnili*.
5. *Embadomonas intestinalis*.

Ova of *Hymenolepis nana* were also present. Stool samples from the same patient, examined in autumn, contained also cysts of the small race of *Entamoeba histolytica* measuring 6–8 μ in diameter. There were also typical uninucleate cysts of *E. histolytica* (large race), the nucleus of which contained a minute karyosome. The dimensions of the *polecki* type of cysts in stained preparations varied from 8·8 to 16·2 μ, with a mean of $12{\cdot}29 \pm 0{\cdot}17\,\mu$, which coincides fairly well with the figures of Kessel & Johnstone (variations from 8 to 15 μ with a mean about 12 μ). The living cysts were about 15% larger,

varying from 10·3 to 17·6 μ, with a mean of 14·20 ± 0·24 μ. The corresponding variation curves, each based on 200 measurements are shown in Fig. 9. Practically all the cysts were spherical; elongated cysts, for example measuring 17 by 13 μ, were rare exceptions. In stained preparations all the cysts were uninucleate with a big spherical nucleus containing a large karyosome (see Figs. 1–8). The diameter of the nucleus is about one quarter that of the cysts. The large central karyosome consists of numerous aggregated granules and is spherical or stellate in shape. Its diameter is about half that of the nucleus. The karyosome is connected with the nuclear membrane by radial achromatic fibres. Numerous small chromatin granules form a layer on this membrane, so that it appears in optical section as a dark ring. Sometimes the granules form a much thicker layer near one pole of the nucleus, thus giving to the nuclear membrane the appearance of a crescent (Fig. 7). In such cases the karyosome can sometimes be eccentric (Fig. 8). Among the many thousands of cysts which were examined, only one binucleate cyst was found. The cytoplasm of nearly all the cysts contained a peculiar dark stained



Figs. 1–8. Cysts of the *polecki* type. Figs. 1, 4. Typical cysts, containing a single nucleus with large karyosome, 'inclusion mass' and numerous chromatoid bodies. Fig. 2. Large cyst containing only three chromatoid bodies. Fig. 3. Cyst, showing 'equatorial belt' of chromatoid bodies arranged in a wide band along the equator of the cyst. Fig. 5. A very small cyst. Fig. 6. Optical section through cyst, showing peripheral position of chromatoid bodies. The nucleus, placed on the lower pole of the cyst, is not shown. Fig. 7. Cyst with only two chromatoid bodies and crescent-shaped accumulation of chromatin on the nuclear membrane. Fig. 8. Cyst with globular chromatoid bodies and eccentric karyosome (degenerate form?).

'inclusion mass', which appears to be a condensation of the cytoplasm in the middle of the cyst (Figs. 1–6). It is more or less spherical in shape, with outlines not sharply defined, and is similar to the 'masse chromophile' depicted by Brumpt (1949) in typical cysts of *E. histolytica*. This inclusion mass is surrounded by a light foamy cytoplasm, containing the nucleus and chromatoid bodies. The latter are small and numerous. Their dimensions very rarely reach half the diameter of the cyst; usually they are from 1 to 4 μ long. With their rounded or tapering—but never threadlike—ends, they lie in the outer layer of cytoplasm, usually parallel to the cyst wall. In freshly passed stools obtained in spring, their number varied from 2 to 20 with a mean of 15. In cysts found in autumn, the number of chromatoid bodies was much less, while a considerable percentage of cysts possessed no chromatoid bodies at all. In fresh or iodine-stained preparations, the *polecki* type of cysts are recognizable owing to the presence of an outer cytoplasmic layer, filled with highly refractile chromatoid bodies and containing a single large nucleus. The inclusion mass is seen in the middle of the cysts. In iodine-stained preparations it never takes on the dark brown colour characteristic of glycogen.

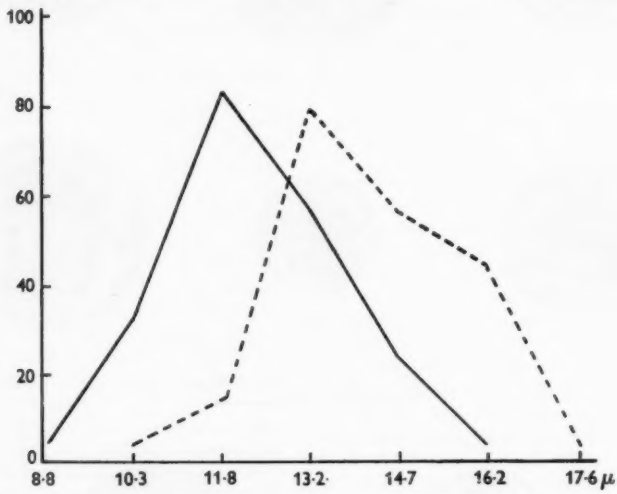


Fig. 9. Dimensions of the cysts of *polecki* type. ——, fixed cysts; – – – –, fresh cysts.

After finding the *polecki* type of cysts in solid stools, I expected to discover some peculiar trophozoites in fluid stools. The examination of fresh stools, obtained after the administration of salt purgative, revealed a trivial fauna of amoebic trophozoites with the prevalence of those identical with the *minuta* form of *Entamoeba histolytica*. These were from 8·5 to 20 μ in diameter when rounded. Their coarsely vacuolated rigid endoplasm always retained its approximately spherical shape, even in trophozoites with long outstretched pseudopodia. The nucleus was typical of *E. histolytica*. Only when it was compressed by voluminous food-vacuoles, did it assume an ellipsoidal shape and the karyosome became eccentric. Eccentric karyosomes were observed also in some trophozoites without food-vacuoles; these were probably precystic forms. Nuclei with peripheral chromatin and an uneven layer of coarse granules on the nuclear membrane, as depicted by Kessel & Johnstone (1949, Fig. 1), were never seen. The food-vacuoles of these trophozoites were filled with bacteria and lower fungi—the usual food of the *minuta* form of *E. histolytica* (Hoare, 1949). Ingested erythrocytes were not observed.

To decide by which kind of trophozoites the *polecki* type of cysts were formed, differential counts of trophozoites in fluid stools and of cysts in solid stools, passed shortly after the administration of purgatives, were performed. The results, obtained on 24 April, are summarized below:

Trophozoites		Cysts	
Entamoeba coli	9 %	*E. coli*	11 %
E. histolytica	72 %	*'polecki'*	67 %
Iodamoeba bütschlii	15 %	*I. bütschlii*	19 %
Endolimax nana	4 %	*E. nana*	3 %

On re-examining the patient in November and December 1950, the *polecki* type of cysts were found again, but this time together with typical small

cysts of *E. histolytica*, measuring from 5·5 to 8 μ in diameter. Differential counts of 300 specimens of both cysts and trophozoites were made in November with the following results:

Trophozoites		Cysts	
Entamoeba coli	17·3 %	*E. coli*	13·0 %
E. histolytica	66·0 %	*polecki* type	33·6 %
Iodamoeba bütschlii	13·7 %	*E. histolytica* (small race)	27·4 %
Endolimax nana	3·0 %	*I. bütschlii*	22·3 %
		E. nana	3·7 %

In another stool sample, examined in the same way, the figures differed from those shown above, but the *E. histolytica* trophozoites were much more numerous than those of *E. coli* and the cysts of *polecki* type were also present in much larger numbers than the *E. coli* cysts.

DISCUSSION

I have failed to find in this material any special type of trophozoites to which the formation of *polecki* type of cysts could be attributed. The source of their formation could be the trophozoites either of *E. coli* or of *E. histolytica*.* If the *polecki* cysts are attributed to *E. coli*, it must be assumed that at least in the material obtained in the spring the much more numerous *E. histolytica* trophozoites did not form cysts at all. On the other hand, there is a striking correspondence between the number of *polecki* cysts and that of *E. histolytica* trophozoites. The latter can, therefore, be regarded as the source from which these cysts have originated, unless it is assumed that the *polecki* cysts belong to a distinct species of *Entamoeba*, with trophozoites morphologically indistinguishable from those of the *minuta* form of *E. histolytica*.

It has already been noted above that the stools contained a small number of *typical* uninucleate cysts of *E. histolytica*; furthermore, there were also some cysts with a nuclear structure which was intermediate between these and the *polecki* type. It is therefore conceivable that in our case a strain of *E. histolytica* was present in which the cysts, for some unknown reason, failed to develop beyond the uninucleate stage and finally degenerated, assuming the appearance seen in the *polecki* cysts. An analogous case of amoebiasis, in which abnormal uninucleate cysts were observed, was recorded by Dobell (1919).

* The *polecki* type of cysts cannot be regarded as belonging to coprozoic protozoa, because (1) they were abundant in fresh solid stools; (2) they were very scarce or absent in fresh fluid stools containing trophozoites; and (3) they were passed by the patient for at least one year, having been found by me again in March 1951.

SUMMARY

1. The occurrence of cysts of the *polecki* type in an apparently healthy person in Pakistan is recorded.

2. The possible relationship of the *polecki* type cysts and the *minuta* form of *E. histolytica* is discussed.

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FAILURE TO PRODUCE RESISTANCE TO CHLOROQUINE IN *PLASMODIUM GALLINACEUM* IN CHICKS

By ANN BISHOP* and ELSPETH W. McCONNACHIE
From the Molteno Institute, University of Cambridge

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I. INTRODUCTION

Since the discovery by Bishop & Birkett (1947) and Williamson, Bertram & Lourie (1947) that strains of *Plasmodium gallinaceum*, maintained in young chicks, could be made fully resistant to proguanil (paludrine) by treatment with that drug, and that resistance is retained after passage through the intermediate host, *Aëdes aegypti*, a new problem has arisen in the chemotherapy of malaria. This resistance to proguanil was induced experimentally in strains of avian malaria maintained in the laboratory under conditions believed to be most suited to the development of drug resistance, but, more recently, reports have been forthcoming of the appearance of proguanil-resistant strains of malaria in man under normal conditions of treatment. Thus, cases of *Plasmodium falciparum*, which no longer responded to treatment with proguanil, have been described by Edeson & Field (1950) from the Tampin area of Malaya. In 1947, when treatment with the drug was begun in this area, clinical response to a single dose of 100–300 mg. of proguanil was good, whereas by the end of 1948 this dose failed to control the attack in a number of cases, and in 1949 even the standard therapeutic course of 300 mg. daily for 7–10 days failed in seven cases. Moreover, Van Goor & Lodens (1950) have reported that in the Tjitamiang district of the Dutch East Indies, the local strain of *P. malariae* has become resistant to a daily dose of 400 mg. of proguanil and the local strain of *P. falciparum* has become resistant to a daily dose of 50 mg. The sensitivity of *P. vivax* to the drug was unaltered. No details of these observations in the Dutch East Indies were given.

Although, hitherto, the development of drug-resistant strains of malaria has not been a problem in the treatment of the disease, these observations suggest that it may become one in the future. Whereas in the past stability, chemotherapeutic index and toxicity were the most important factors in assessing the qualities of a new antimalarial, in future its power to produce resistant strains of parasite may also have to be taken into consideration.

There is some evidence that strains of malaria within a species may vary in their ability to become resistant to drugs. Thus Williamson & Lourie (1947) failed to obtain strains of *P. gallinaceum* resistant to sulphadiazine or quinine, whereas Bishop & McConnachie (1950) succeeded in developing strains resistant to the former drug, and Knoppers (1947) produced a strain resistant to quinine, although the degree of resistance attained was only twofold. In addition, it has been shown by Rollo, Williamson & Lourie (1948) and McConnachie (1951) that the conditions under which a strain is maintained whilst in contact with the drug are important in determining whether resistance will develop. Williamson & Lourie (1947) obtained resistance to proguanil in 2½–3½ months when strains of *P. gallinaceum* maintained in a state of acute infection were treated with the drug, but no resistance developed when the strain was in a latent state throughout the period of treatment (Rollo *et al.* 1948). Bishop & McConnachie (1950) not only obtained strains of *P. gallinaceum* resistant to sulphadiazine when acute infections of this organism were treated with the drug, but these strains proved to be cross-resistant to proguanil. When, however, latent infections were treated with sulphadiazine for over 6 months no resistance either to sulphadiazine or to proguanil developed (McConnachie, 1951). In comparing the potentiality of antimalarial drugs for producing resistant strains, it is essential, therefore, that the strains exposed to the drugs should be identical in type and history. In recent years chloroquine (7-chloro-4-(4-diethylamino-1-methylbutylamino) quinoline, SN 7618, resorchin, nivaquine B) has been introduced for the treatment of malaria and appears to have certain advantages over mepacrine (Loeb *et al.* 1946; Pullman, Craige, Alving, Whorton, Jones & Eichelberger, 1948;

* Member of the Scientific Staff of the Medical Research Council.

Berliner, Earle, Taggart, Zubrod, Welch, Conan, Bauman, Scudder & Shannon, 1948; Findlay, 1951). As this compound may be used extensively in the future in the treatment of this disease, it is important to know whether it readily produces resistant strains of malaria. We have, therefore, treated a substrain of the parent strain of *P. gallinaceum*, from which in the past our resistant strains have been derived, with chloroquine for a period of 16 months, under conditions similar to those by which resistance to paludrine, sulphonamides and pamaquin have been developed.

II. EXPERIMENTS

In attempting to develop resistance to a drug in strains of *P. gallinaceum* it has been our practice (Bishop & Birkett, 1947, 1948; Bishop & McConnachie, 1950) to transmit the strain from chick to chick, by intravenous injection of infected, heparinized blood, at intervals of a few days. In the early stages of such experiments the inoculum used is relatively large (0·5–1·0 c.c.), but it is gradually reduced as drug resistance begins to develop. The strain of *P. gallinaceum* treated with chloroquine has been maintained in this way. The drug has been administered as the diphosphate, orally in water, to chicks 45–65 g. in weight. In developing drug-resistant strains of *P. gallinaceum* we have found that resistance arises most readily when, in the early stages of treatment, the dose of drug is so small that it has little effect upon the parasite; as resistance develops the dose can gradually be increased. In our attempts to obtain a strain of *P. gallinaceum* resistant to chloroquine the first dose used was 0·04 mg./20 g. of body weight once daily,* but as this had no effect upon the parasites it was increased to 0·08 mg. once daily, which also had no apparent effect. After 6 weeks' treatment with these doses, 0·16 mg. was given once daily. The number of parasites found in birds receiving this dose was small and they were all abnormal in appearance. In order that large numbers of parasites should be exposed to the action of the drug it was necessary to pass the strain alternately through birds receiving 0·16 mg. of chloroquine once daily, and birds receiving no drug. After this treatment had been continued for approximately 6 months the proportion of abnormal parasites decreased, and it was no longer necessary to pass the strain through untreated birds in order to maintain heavy infections; this suggested that the strain might have become slightly less sensitive to the drug. Its sensitivity to chloroquine, therefore, was compared with that of the normal stain.

* Throughout this paper doses are expressed in mg./20 g. of body weight of bird.

In comparing the sensitivity of the normal and chloroquine-treated strains to chloroquine, the method described previously was used (Bishop & Birkett, 1948). Briefly, this method consists of inoculating each of a batch of chicks, 45–65 g. in weight, with 50,000,000 parasites of the chloroquine-treated strain per bird intravenously. Five of these birds received no drug and served as controls, whereas other groups of five were treated with the doses of drug to be tested, each group receiving a different dose. A second batch of chicks was inoculated with the normal parent strain and treated similarly. A total of four doses of chloroquine was given, the first being given immediately before the chicks were inoculated, and the remaining three once daily on the three succeeding days. The intensity of infection was assessed from blood films made on the fourth morning following inoculation, and is expressed as the mean number of parasitized corpuscles per 500 corpuscles in a group of five birds. The intensity of infection produced by the chloroquine-treated and parent strains in birds receiving similar doses of drug was compared.

It was obvious from the infections produced by the chloroquine-treated and normal parent strains in birds receiving 0·08 mg. of chloroquine, that this dose has little effect upon either strain, as they were of the same order of intensity as the untreated controls (Table 1). Doses of 0·16 and 0·2 mg. of chloroquine once daily produced a slightly inhibitory effect upon the growth of the chloroquine-treated strain and a more marked effect upon the normal parent strain. This result suggested that the strain might be developing a slight degree of resistance to the drug; but results based upon one experiment with doses of drug which show only slight antimalarial activity cannot be considered to be significant, as in birds receiving such doses the intensity of infection is always variable.

In order to try to stimulate the development of resistance the dose of chloroquine was increased to 0·2 mg. once daily. This slight increase in dose produced a very marked effect upon the parasites; they became vacuolated and abnormal in appearance, and in order that the blood used for the inocula should be heavily parasitized it was again necessary to pass the strain through untreated birds from time to time to increase the intensity of infection.

After the strain had been maintained in this way for a total period of 16 months, during which it had been passaged eighty-nine successive times and exposed to an aggregate dose of 52·64 mg./20 g. of chloroquine, its sensitivity to the drug was again assessed. In these tests, unlike the former, the doses were given twice daily for 3½ days, the first dose being given immediately before inoculation and the remaining six twice daily on three successive days. The lowest dose, 0·08 mg. twice daily, had a marked

though not complete antimalarial action upon the normal and the chloroquine-treated strains, the numbers of surviving parasites varying in different birds. No significant difference between the two strains was observed in birds receiving this dose. Higher doses (0·16 and 0·2 mg. twice daily) inhibited the growth of both the normal and chloroquine-treated strains (Table 1).

through birds receiving 0·16 mg. of chloroquine once daily the proportion of badly affected parasites slowly decreased and the intensity of the infections produced in the presence of the drug increased. This change, however, was not sufficiently marked to give clear-cut results when relatively small numbers of parasites were exposed to the drug in the test.

Table 1. *Comparison of intensity of infections produced by chloroquine-treated and parent strains of* Plasmodium gallinaceum *in chicks treated with chloroquine, mepacrine or quinine*

Strain	No. of passages through drug-treated birds	Control	Dose in mg./20 g. of body weight: Chloroquine diphosphate 0·4*	0·2	0·2*	0·16	0·16*	0·08	0·08*	Mepacrine 0·64*	0·32*	Quinine 0·8	0·4
Chloroquine	29	258	0	—	121	—	195	—	269	—	—	—	—
Parent		264	0	—	45	—	125	—	231	—	—	—	—
Chloroquine	89	188	—	1	—	1	—	—	—	6	—	—	—
Parent		252	—	0	—	0	—	—	—	1	—	—	—
Chloroquine	89	156	—	—	—	0	—	7	—	1	96	12	50
Parent		279	—	—	—	0	—	11	—	0	76	0	51

* Doses given once daily.

The intensity of infection is expressed as the average number of infected erythrocytes per 500 erythrocytes in groups of four to five birds.

Both from the behaviour of the strain while undergoing drug treatment and from these results, it must be concluded that in spite of an exposure to chloroquine of 16 months, this strain of *P. gallinaceum* had failed to develop any significant degree of resistance to the drug.

As we had found previously (Bishop & McConnachie, 1950) that in birds treated with sulphadiazine, resistance to proguanil (paludrine) may develop more rapidly than resistance to sulphadiazine itself, we thought it of interest to find out whether prolonged treatment with chloroquine had altered the sensitivity of the strain to such drugs as mepacrine and quinine. However, the infections produced by the chloroquine-treated strain in birds treated with the minimum effective doses of mepacrine and quinine did not differ significantly from those of the normal parent strain under similar conditions (Table 1). The reaction of the chloroquine-treated strain to these drugs, therefore, had not changed during the prolonged period of treatment.

III. DISCUSSION

When a population of *P. gallinaceum* in chicks is exposed to low doses of chloroquine, the reaction of individual parasites to the drug varies considerably. Some are killed rapidly, some are affected more slowly and show vacuolated cytoplasm, clumped pigment and abnormal nuclei, whereas others are able to grow and multiply though their cytoplasm may be vacuolated. When the strain was passed

When the dose was increased to 0·2 mg. once daily the proportion of parasites killed or badly affected was very great, though it varied in different birds, and less badly affected parasites were always present; but in spite of continuing drug treatment for a period of 10 months no significant change in the proportions took place. It must therefore be concluded that the less badly affected parasites do not give rise only to parasites as little affected by the drug as themselves; if this were so, the population would rapidly change into one consisting entirely of slightly affected individuals, whereas, even after months of treatment with 0·2 mg. of the drug the greater part of the population still consisted of badly affected or dead parasites. This large proportion of badly affected parasites could not have been derived from a reserve of exo-erythrocytic parasites unaffected by the drug, as the passage of the strain at intervals of a few days precluded the development of a large number of exo-erythrocytic forms; they must therefore have been derived from parasites not sufficiently affected to inhibit multiplication.

Several workers have shown that resistance to proguanil develops relatively rapidly in *P. gallinaceum*, and it has also been shown that the ability to form resistant strains is not limited to this species of malaria. Hawking & Perry (1948) and Schmidt, Genther, Fradkin & Squires (1949) obtained proguanil-resistant strains of *P. cynomolgi* in monkeys, whilst Adams & Seaton (1949) have obtained a proguanil-resistant strain of *P. falciparum*, West African in

origin, and maintained by blood inoculation, and Lourie & Seaton (1949) obtained similar results with *P. vivax*. More recently, Cooper, Coatney & Imboden (1950) have treated the Chesson strain of *P. vivax* with proguanil and obtained a highly resistant strain. The production of proguanil-resistant strains in malaria organisms therefore appears to be relatively easy. With mepacrine, however, all attempts to produce resistant strains under experimental conditions have so far failed; thus Bishop & Birkett (1947) failed to obtain a strain of *P. gallinaceum* resistant to mepacrine after 6 months' intensive treatment with the drug; Williamson & Lourie (1947) treated a strain of the same species with mepacrine for 27 months without producing resistance, and Schmidt *et al.* (1949) also failed to produce a mepacrine-resistant strain of *P. cynomolgi*. Evidence is also accumulating which indicates that chloroquine does not form resistant strains of malaria, even after intensive treatment with the drug. Thus Thompson (1948) failed to obtain resistance either to chloroquine or to the related 4-aminoquinoline camoquin when he treated *P. lophurae* with these drugs for 200 days; Schmidt *et al.* (1949) also failed with *P. cynomolgi*, and Cooper *et al.* (1950) with the Chesson strain of *P. vivax*. As these workers all obtained resistance to proguanil quite readily in the same species of malaria it is to be concluded that there is a fundamental difference between this drug and chloroquine in ability to form resistant strains.

The chemical relationship of the 4-aminoquinolines with the acridines, which can be regarded as 2, 3-benzoquinolines, has been pointed out by Blanchard (1947). Loeb and his co-workers (1946) have shown that pharmacologically chloroquine and mepacrine resemble one another; and Black (1946) and Thompson (1948) have observed that these drugs have a similar effect upon the malaria parasite, which suggests that their mode of action may be similar. Sulphadiazine and proguanil exert their action upon the same stage of the malaria parasite, by inhibiting division, and these drugs also resemble each other in forming drug-resistant strains readily.

The inability of mepacrine and chloroquine to form resistant strains of malaria parasite, as shown experimentally, may prove an important factor in the choice of a drug for large-scale use in the treatment of malaria.

IV. SUMMARY

A strain of *Plasmodium gallinaceum* has been treated for 16 months with chloroquine, under conditions which previously have produced strains resistant to proguanil, sulphonamides and pamaquin, but no change in reaction to the drug has occurred.

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PAMAQUIN RESISTANCE IN A STRAIN OF *PLASMODIUM GALLINACEUM* AND ITS RELATIONSHIP TO OTHER ANTIMALARIAL DRUGS

BY ANN BISHOP* AND ELSPETH W. McCONNACHIE
From the Molteno Institute, University of Cambridge

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I. INTRODUCTION

In 1934 Nauck described the occurrence of resistance to quinine and pamaquin† in relapsed infections of *Plasmodium knowlesi* in *Macacus rhesus*, after treatment with these drugs. Later, Fulton & Yorke (1941) obtained a pamaquin-resistant strain of the same species of malaria. In their experiments resistance first appeared 2 months after the commencement of pamaquin treatment and increased with the dosage, until finally the parasites failed to respond to the maximum amount of drug which the macaque would tolerate. The total enhancement of resistance, however, was only fourfold. Fulton (1942) failed to produce resistance to pamaquin in *Plasmodium gallinaceum* in fowls, but Bishop & Birkett (1947, 1948) obtained a strain resistant to the minimum effective dose as a result of 8 months' treatment with the drug. This strain has been maintained by us and, in the intervening years, has undergone further treatment with pamaquin. Its reaction to other drugs has been studied.

II. METHODS

The strain of *P. gallinaceum* undergoing treatment with pamaquin has been maintained in chicks, 45–65 g. in weight, by intravenous passage of heparinized blood at intervals of 2–3 days. For comparison, the untreated parent strain has been maintained in the same way. The drug was given orally, by catheter tube, into the stomach of the bird. The dose was proportional to the body weight and is expressed in mg./20 g. of body weight of chick.

The sensitivity of the pamaquin-treated strain to pamaquin and other antimalarial drugs has been tested by the method described previously (Bishop & Birkett, 1947, 1948). The required number of chicks was inoculated intravenously with approximately 50,000,000 parasites of the pamaquin-treated strain per bird; five of these birds received no drug and served as controls, whereas other groups of five were treated each with a different amount of pamaquin or other drug. The drug was administered once or twice daily, for 3½ days, the first dose being given immediately before inoculation. Blood films were made on the fourth day following inoculation and the intensity of infection was assessed as the number of parasitized erythrocytes per 500 erythrocytes. The sensitivity of the untreated parent strain was tested in the same way for comparison with the pamaquin-treated strain.

III. THE DEVELOPMENT OF RESISTANCE TO PAMAQUIN IN A STRAIN OF *PLASMODIUM GALLINACEUM*

In their attempts to produce resistance to pamaquin in a strain of *P. gallinaceum*, Bishop & Birkett (1948) first treated the chicks through which the strain was passed with 0·01 mg. of the drug once daily. This was the highest dose which had no apparent effect upon the parasites when they were subjected to it for a long period. The dose was gradually increased through 0·02, 0·04 to 0·08 mg. The effect of 0·08 mg.

* Member of the Scientific Staff of the Medical Research Council.

† Pamaquin, plasmoquine and plasmochin are synonyms under which the compound 6-methoxy-8-diethylamino-*iso*pentylaminoquinoline is marketed.

upon the parasites was at first pronounced, but it gradually diminished, and finally ceased to be apparent after the strain had been passed for a prolonged period through birds receiving this amount of the drug. After 5 months' treatment with this dose, a good growth of healthy parasites was obtained in the drugged birds. The sensitivity of the pamaquin-treated and normal parent strains to once-daily doses of 0·08 mg. of pamaquin was compared and the pamaquin-treated strain was found to be definitely less sensitive than the parent strain (Bishop & Birkett, 1948, Table 2). The strain was thus shown to have developed a low-grade resistance to pamaquin.

When the dose of pamaquin was increased to 0·08 mg. twice daily the strain was again severely affected; it grew very poorly and the majority of the parasites were abnormal in appearance; indeed its growth was so poor that it was necessary, from time to time, to pass it through untreated birds in order to keep the infections in a patent state. It was not until the parasites had been in contact with this increased amount of drug for 6 months that the strain grew well in birds receiving this dose. An increase in the dose to 0·16 mg. once daily produced little effect either upon the appearance of the parasites or their growth rate, but they became severely affected when this dose was given twice daily, and again it became necessary occasionally to pass the strain through untreated birds in order to keep it in a patent state. After 8 months' treatment with 0·16 mg. of pamaquin twice daily the strain grew well in birds receiving this dose. The intensity of infections produced by the pamaquin-treated strain in birds treated with these higher doses of drug was then assessed by the method already described.

Although, at the time when the first tests were made, the strain had been maintained without difficulty for over 1 year in birds receiving 0·16 mg. of pamaquin twice daily, the infections produced in birds receiving this dose once, or twice daily were less heavy than in untreated birds or in birds receiving lower doses of pamaquin (Table 1). The parasites were, however, normal in appearance. In birds treated with 0·04 and 0·08 mg. of pamaquin twice daily, doses which, like 0·16 mg., inhibited the growth of the parent strain, heavier infections were obtained.

Not only were the infections produced by the pamaquin-treated strain in birds receiving the higher doses of drug lighter than those in untreated birds, but, on the whole, the latter were less heavy than similar untreated infections of the parent strain. It must be concluded that, in spite of the long period during which the pamaquin-treated strain had been in contact with the drug, it had not become completely adjusted to pamaquin and even in untreated birds its growth was slower than that of the untreated parent strain.

No attempt was made to produce resistance to larger doses of pamaquin than 0·16 mg. twice daily.

Throughout the period of treatment with pamaquin, gametocytes were few or absent; even when the strain had been maintained for a long period in birds receiving twice daily doses of 0·16 mg. of pamaquin and healthy schizonts were numerous, gametocytes were still rare. When, however, the pamaquin-resistant strain was inoculated into untreated birds, gametocytes of normal appearance became relatively numerous. This suggests that the strain had developed greater resistance to the schizonticidal action of the drug than to the gametocidal action, and not that it had lost the power to produce gametocytes.

IV. THE SENSITIVITY OF THE PAMAQUIN-RESISTANT STRAIN TO OTHER ANTIMALARIAL DRUGS

The study of cross-resistance between compounds of therapeutic importance is a useful method of discovering related modes of action. Similarity in the chemical structure of the drugs is not essential to cross-resistance; thus strains of *P. gallinaceum* made resistant to sulphadiazine or sulphanilamide by treatment with those drugs were found to be highly resistant to proguanil* (Bishop & McConnachie, 1948, 1950*a*, *b*). Tests for cross-resistance to other compounds of proved antimalarial action were therefore made with the pamaquin-resistant strain in order to ascertain whether the mode of action of pamaquin is related to that of any of the well-known antimalarial compounds. In these tests the sensitivity of the pamaquin-resistant strain was assessed against the minimum dose of drug which had an inhibitory effect upon the untreated normal strain under the conditions of the test. If the minimum effective dose (M.E.D.) of a drug for the normal strain of *P. gallinaceum* was not already known it was determined. With some compounds, such as sulphadiazine, where the M.E.D. is not sharply defined, the pamaquin-resistant strain was tested against the lower doses which reduced the intensity of infection of the parent strain significantly.

* Proguanil (paludrine, chlorguanide): *N'*-*p*-chlorophenyl-N_5-*iso*propylbiguanide.

Sulphadiazine: 2-(*p*-aminobenzenesulphonamido)-pyrimidine.

Mepacrine (atebrin, atebrine, quinacrine): 6-chloro-9-(4-diethylamino-1-methylbutylamino)-2-methoxyacridine.

Chloroquine (resorchin, nivaquine, nivaquine B, aralen, SN. 7618): 7-chloro-4-(4-diethylamino-1-methylbutylamino) quinoline.

Sontochin (Sontoquine, SN. 6911): 7-chloro-4-(4-diethylamino-1-methyl-butylamino)-3-methylquinoline.

Pentaquine (SN. 13276): 8(5-*iso*propylaminoamylamino)-6-methoxyquinoline.

Table 1. *Comparison of intensity of infections produced by the pamaquin-resistant and parent strains of* Plasmodium gallinaceum *in chicks treated with pamaquin, quinine and pentaquine*

		Dose in mg. per 20 g. of body weight									
		Pamaquin				Quinine			Pentaquine		
Strain	Control	0·16	0·16*	0·08	0·04	1·6	0·8	0·4	0·02	0·01	0·005
Pamaquin	289	56	—	—	—	—	—	—	—	—	—
Parent	347	0	—	—	—	—	—	—	—	—	—
Pamaquin	263	77	154	—	—	—	88	131	—	—	—
Parent	387	0	0	—	—	—	5	171	—	—	—
Pamaquin	206	83	—	181	211	105	236	—	—	—	—
Parent	286	—	—	0	12	—	8	—	—	—	—
Pamaquin	286	140	—	309	221	22	189	—	—	—	—
Parent	393	—	—	1	31	—	15	—	—	—	—
Pamaquin	102	—	—	—	—	—	—	—	36	169	248
Parent	385	—	—	—	—	—	—	—	—	1	93
Pamaquin	156	—	—	—	—	—	—	—	66	106	—
Parent	413	—	—	—	—	—	—	—	1	5	—
After passage of the pamaquin-resistant strain through untreated birds for 6 months											
Pamaquin	269	14	—	241	307	—	—	—	—	—	—
Parent	312	—	—	—	15	—	—	—	—	—	—
Pamaquin	187	—	—	139	213	—	111	—	—	—	—
Parent	306	—	—	0	20	—	1	—	—	—	—

* Dose given once daily.

The intensity of infection is expressed as the average number of infected erythrocytes per 500 erythrocytes in groups of 4 to 5 birds.

Table 2. *Comparison of intensity of infections produced by the pamaquin-resistant and parent strains of* Plasmodium gallinaceum *in chicks treated with mepacrine, chloroquine, sontochin, proguanil and sulphadiazine*

		Dose in mg./20 g. of body weight								
		Mepacrine		Chloroquine	Sontochin		Proguanil	Sulphadiazine		
Strain	Control	0·64*	0·32*	0·08	0·32	0·16	0·1	1·25	0·625	0·3125
Pamaquin	219	16	239	—	—	—	—	—	—	—
Parent	347	1	289	—	—	—	—	—	—	—
Pamaquin	263	5	170	—	—	—	—	—	—	—
Parent	387	0	332	—	—	—	—	—	—	—
Pamaquin	206	—	—	—	—	—	—	5	10	—
Parent	286	—	—	—	—	—	—	10	14	—
Pamaquin	286	—	—	38	—	—	—	—	10	87
Parent	393	—	—	68	—	—	—	—	13	112
Pamaquin	192	—	—	—	1	18	—	—	—	—
Parent	243	—	—	—	1	37	—	—	—	—
Pamaquin	215	—	—	—	—	—	0	—	—	—
Parent	340	—	—	—	—	—	0	—	—	—

* Dose given once daily.

The intensity of infection is expressed as the average number of infected erythrocytes per 500 erythrocytes in groups of 4 to 5 birds.

The pamaquin-resistant strain showed no resistance to proguanil, sulphadiazine, mepacrine, chloroquine or sontochin (Table 2); but when it was tested against pentaquine and quinine its sensitivity was found to be less than that of the untreated parent strain (Table 1). Parasites of normal appearance were numerous in birds infected with the pamaquin-resistant strain and treated with 0·8 mg. of quinine twice daily for 3½ days, whereas few parasites of the parent strain survived this treatment and these were severely affected by the drug. Although the infections produced by the pamaquin-resistant strain in birds receiving 1·6 mg. of quinine twice daily were slighter than those in birds receiving 0·8 mg. of the drug, and the intensity varied considerably from bird to bird, the parasites did not appear to be affected by the drug. In birds receiving 3·2 mg. of quinine no growth of parasites occurred. The pamaquin-resistant strain also produced infections of parasites of normal appearance in birds treated with the lower range of suppressive doses of pentaquine. Thus the development of resistance to pamaquin conferred some resistance to both quinine and pentaquine.

V. THE EFFECT UPON RESISTANCE TO PAMAQUIN AND QUININE OF MAINTAINING THE PAMAQUIN-RESISTANT STRAIN IN THE ABSENCE OF THE DRUG

It has been shown (Bishop & McConnachie, 1950*c*) that a strain of *P. gallinaceum* made resistant to proguanil retained its resistance undiminished for over a year in the absence of the drug. During this period it was maintained in a state of acute infection in untreated chicks, by passage at frequent intervals. These experiments suggest that resistance to proguanil, once acquired, is a very stable quality.

Resistance to pamaquin in *P. gallinaceum* developed much more slowly than resistance to proguanil, though the experimental conditions were similar. Resistance to proguanil became complete in about 4½ months, whereas resistance to pamaquin was not complete even after the strain had been maintained in contact with the drug for several years. It was, therefore, of interest to discover whether pamaquin-resistance was as stable as proguanil-resistance.

A substrain of the pamaquin-resistant strain was tested for resistance to 0·16 mg. of pamaquin twice daily and found to be resistant to this dose. The strain was then passaged, by intramuscular injection, at intervals of approximately 8 days, for a period of 6 months, through chicks receiving no drug. At the end of this period the sensitivity of the strain to pamaquin was again tested (Table 1). It was found to have retained its resistance to 0·08 and 0·04 mg. twice daily, but its resistance to 0·16 mg. had been almost completely lost. In birds treated with this dose only a few parasites, mostly abnormal in appearance, were found.

As an appreciable loss in resistance to pamaquin was found to have occurred, the sensitivity of the strain to quinine was also tested. Resistance to the M.E.D. (0·8 mg.) still persisted. Although the infections produced by the pamaquin-resistant strain in birds treated with this dose were not heavy, the parasites were normal in appearance.

VI. THE EFFECT OF PAMAQUIN AND QUININE IN COMBINED DOSES UPON *PLASMODIUM GALLINACEUM*

It was observed by Greenberg, Boyd & Josephson (1948) that the action of proguanil and sulphadiazine, when given concurrently, was synergistic. The effective dose of proguanil could be reduced to $\frac{1}{4}$ if combined with $\frac{1}{32}$ to $\frac{1}{64}$ the effective dose of sulphadiazine. Antimalarial compounds other than sulphadiazine were also found to synergize with proguanil, but these drugs, like sulphadiazine, were all inhibitors of *p*-aminobenzoic acid (Greenberg, 1949*b*).

Bishop & McConnachie (1948, 1950*a*) had found that strains of *P. gallinaceum* made resistant to sulphadiazine were likewise resistant to proguanil. The question therefore arose whether all antimalarial drugs which produce cross-resistance likewise synergize with one another. If the ability of drugs to produce cross-resistance and to synergize is related, then as pamaquin has been shown to produce cross-resistance to quinine, these two drugs should synergize in their antimalarial action.

More than 20 years ago Sinton & Bird (1928) observed that the combination of quinine with pamaquin was a distinct advantage over either of the drugs used alone, and Sinton, Smith & Pottinger (1930) found that a higher percentage of radical cures was obtained by giving small doses of the drugs concurrently than by larger doses given separately. Bratton (1945) compared the effect of antimalarial compounds given separately or concurrently upon infections of *P. lophurae* in ducks, and did not find any summation with quinine and pamaquin. He concluded that the principal mechanism of antimalarial action of these two compounds is different. More recently, Reilly, Chen & Geiling (1950) have described a synergistic effect in the suppressive action of quinine and pamaquin, and quinine and pentaquine upon *P. lophurae* when these drugs are combined. Further investigation of this problem seemed desirable.

In testing a combination of antimalarial drugs for synergism of their suppressive action, it is necessary to use doses smaller than those which

produce full suppression of the parasites with either drug alone within the period of the test. With the normal strain of *P. gallinaceum* doses of 0·04 mg. of pamaquin reduce the intensity of infection drastically though they do not suppress an infection of 50,000,000 parasites completely when given twice daily for a period of 3½ days. With 0·02 mg., under similar conditions, the results vary from bird to bird but, as a whole, suppression is slight; with 0·01 mg. parasites from the blood of the chickens; throughout the greater period of treatment, therefore, the type of parasite submitted to the action of the drug would have been mainly exo-erythrocytic. Recent work suggests that, in malaria, drug-resistance arises only when erythrocytic parasites, maintained in a state of constant multiplication, are exposed to the action of the drug. Thus, with proguanil, resistance does not develop if the strain of *P. gallinaceum* undergoing

Table 3. *The effect of pamaquin and quinine, given separately or in combination, upon infections of the parent strain of* Plasmodium gallinaceum *in chicks*

Compound	Dose (mg./20 g.)	Average no. of infected erythrocytes per 500 in groups of 4–5 birds		
Control	—	413	417	430
Pamaquin	0·01	285	401	423
	0·02	—	—	267
Quinine	0·2	132	226	401
	0·4	—	—	131
Pamaquin + quinine	0·005 + 0·1	—	421	—
Pamaquin + quinine	0·005 + 0·2	—	349	—
Pamaquin + quinine	0·01 + 0·1	—	379	—
Pamaquin + quinine	0·01 + 0·2	153	343	—
Pamaquin + quinine	0·02 + 0·2	—	—	349
Pamaquin + quinine	0·01 + 0·4	—	—	311

it is frequently not detectable. With quinine in doses of 0·4 mg. twice daily the results vary from almost complete to partial suppression; with 0·2 mg. twice daily slight suppression occurs in some infections but not in others. When the two drugs were given concurrently, twice daily for 3½ days, in doses which when the drugs were given singly gave slight suppression, or in half these doses, no increase in antimalarial activity could be detected (Table 3).

VII. DISCUSSION

Whereas workers have obtained proguanil-resistant strains of avian, monkey and human malaria without difficulty and in relatively short periods of time, attempts to produce resistance to pamaquin in the two former types of parasite have either failed or resulted in only a low grade of resistance to the drug. Thus Fulton (1942) failed to produce resistance to pamaquin in *P. gallinaceum*, although the strain was treated with the drug for a period of 15½ months. In these experiments the amount of drug given was sufficient to cause a temporary disappearance of the treatment with the drug is maintained in a state of latent infection (Rollo, Williamson & Lourie, 1948), and similar results have been obtained with sulphadiazine (McConnachie, 1951). It is possible, therefore, that Fulton's failure to induce resistance to pamaquin was due to conditions of treatment and not to the nature of the strain.

In a strain of *P. knowlesi*, the species in which Nauck (1934) first observed resistance to pamaquin and quinine, Fulton & Yorke (1941) developed resistance to the maximum dose of pamaquin which the host (*Macacus rhesus*) would tolerate, in a little over 2 months, although the total enhancement of resistance was only fourfold. Resistance to pamaquin, therefore, can develop much more rapidly in this species of parasite than in *Plasmodium gallinaceum*. This facility, however, does not extend to *P. cynomolgi*; Schmidt, Genther, Fradkin & Squires (1949) were unable to produce resistance to the 8-aminoquinolines pamaquin, pentaquine and *iso*-pentaquine with this latter species of monkey malaria, though it had readily become resistant to proguanil.

Not only is resistance to pamaquin produced with greater difficulty in malaria parasites than is resistance to proguanil, but it is not as stable a character. Some loss in resistance was observed in *P. knowlesi* after the pamaquin-resistant strain had been maintained for a period of 5½ months, as a chronic infection in a monkey (Fulton & Yorke, 1943); some loss also occurred, during a period of 6 months, when the pamaquin-resistant strain of *P. gallinaceum* was maintained in untreated birds, by passage at frequent intervals (p. 60). No loss in resistance could be detected in a proguanil-resistant strain after it had been maintained in a state of acute infection and in the absence of the drug, for over one year (Bishop & McConnachie, 1950*c*).

When the pamaquin-resistant strain of *P. gallinaceum* was tested for resistance to other drugs of known antimalarial action, resistance to the related 8-aminoquinoline, pentaquine, and to quinine was observed, but no resistance to mepacrine, chloroquine, sontochin, proguanil and sulphadiazine could be detected. Of these compounds quinine, pentaquine, chloroquine and sontochin are, like pamaquin itself, quinoline compounds. Pentaquine differs from pamaquin only in the constitution of its side chain (see footnote, p. 58); it is not remarkable, therefore, that resistance to pamaquin should confer resistance also to that compound. Chloroquine and sontochin are both 4-aminoquinolines, and Blanchard (1947) has stressed the similarity in antimalarial action of these compounds to mepacrine, which he regards as a 2,3-benzoquinoline. Not only do mepacrine and these 4-aminoquinolines resemble one another in their schizontocidal action, but the changes produced in the appearance of the parasite by their action are similar (Black, 1946). Mepacrine and chloroquine were found by Geiman (1948) to affect glucose and lactate utilization in *P. cynomolgi*, whereas pentaquine and *iso*pentaquine did not. Moreover, with neither mepacrine nor chloroquine have resistant strains of malaria parasite been obtained experimentally, though attempts have been made with *P. gallinaceum*, *P. lophurae* and *P. cynomolgi* (Bishop & Birkett, 1947, 1948; Williamson & Lourie, 1947; Thompson, 1948; Schmidt *et al.* 1949; Bishop & McConnachie, 1952). Our observation that the pamaquin-resistant strain shows resistance to the related 8-aminoquinoline pentaquine, but not to mepacrine or the 4-aminoquinoline chloroquine and sontochin, provides additional proof of the difference in mode of action of these two groups of compounds.

Bishop & McConnachie (1948, 1950*a*, *b*) have found that strains of *P. gallinaceum* made resistant to sulphadiazine or sulphanilamide by treatment with those drugs likewise become highly resistant to proguanil, but the relationship between proguanil-resistance and the sulphonamides is not so clearly established. In their preliminary experiments Bishop & McConnachie (1948) found that a strain of *P. gallinaceum* made fully resistant to proguanil was resistant also to sulphadiazine; they have, however, been unable to confirm this observation. Greenberg (1949*a*), on the contrary, found that hypersensitivity to sulphadiazine developed coincidentally with proguanil-resistance in a strain of *P. gallinaceum* treated with proguanil. A similar relationship appears to occur between pamaquin and quinine, although the degrees of cross-resistance and hypersensitivity are much slighter than those conferred by sulphadiazine and proguanil. The pamaquin-resistant strain of *P. gallinaceum* has been found to be resistant to the lower effective doses of quinine, and Knoppers (1949) found that a strain which had developed a twofold increase in resistance to quinine by treatment with that drug, was hypersensitive to the 8-aminoquinolines pamaquin, pentaquine, and *iso*pentaquine, although its sensitivity to mepacrine, chloroquine, proguanil and sulphamerazine was normal.

A further type of relationship between sulphonamides and proguanil has been described; a synergistic effect in antimalarial action has been demonstrated when sulphadiazine and proguanil were given concurrently (Greenberg *et al.* 1948). The ability to synergize with proguanil has been shown to extend to other *p*-aminobenzoic acid competitors, provided that they themselves have antimalarial activity (Greenberg, 1949*b*).

A considerable amount of data has been accumulated upon the combined action of quinine and pamaquin upon human malaria since Sinton *et al.* (1930) first observed that a higher proportion of radical cures could be obtained in benign tertian malaria with smaller doses of quinine and pamaquin given together than with larger doses of either drug given separately. The curative action of pentaquine also has been found to be enhanced by quinine (Loeb, 1946; Alving, 1948), but not by other schizontocides such as proguanil (Alving, Craige, Jones, Whorton, Pullman & Eichelberger, 1948). White, Cooper, Coatney, Culwell, Lints & Young (1948) have produced some experimental evidence that pentaquine, when administered with quinine, acts upon the exo-erythrocytic parasites. When pentaquine and quinine were administered concurrently to patients infected with the St Elizabeth strain of *P. vivax*, during a latent period of the infection, further relapse was prevented, whereas quinine alone failed to do so. As, at the time of administration of the two drugs, circulating erythrocytic parasites could not be detected either by direct observation or by injection of blood into susceptible recipients, the action of pentaquine must be assumed to be confined to the exo-erythrocytic parasites. These observations upon the combined effect of 8-aminoquinolines and quinine are all based upon the pre-

vention of relapse, and are not, therefore, comparable to the synergism in schizontocidal action which has been described by Greenberg and his co-workers in *P. gallinaceum*, for the *p*-aminobenzoic-acid-inhibited antimalarial compounds and proguanil; rather do they appear, as pointed out by Koenig (1949), to be the result of the parallel action of the two drugs, each working upon a different stage of the life cycle of the parasite—the erythrocytic and the exo-erythrocytic stages. Reilly *et al.* (1950) have, however, described a synergistic effect in the suppressive action of quinine and pamaquin, and quinine and pentaquine, upon *P. lophurae*, when these drugs were given concurrently, but we have been unable to confirm this, in *P. gallinaceum*.

Any conjectures upon the mode of action of pamaquin and quinine and the significance of the resistance to quinine conferred by pamaquin, would not be profitable in the present state of our knowledge of these drugs. Evidence from recent experimental work suggests that the inhibitory action of pamaquin upon malaria parasites is exerted by a metabolite and not by the compound itself. Thus no correlation was found to exist between the concentration of the drug in the plasma and its antimalarial effect, whether this be suppressive or curative (Berlinner, Earle, Taggart, Welch, Zubrod, Knowlton, Atchley & Shannon, 1948). According to Zubrod, Kennedy & Shannon (1948) pamaquin is so extensively metabolized in the vertebrate body that little of the drug is excreted in the urine. When added to cultures of *P. gallinaceum* pamaquin did not possess the marked antimalarial action associated with it *in vivo* (Greenberg, Josephson & Taylor, 1951). In chickens treated with pamaquin no measurable amount of the drug was detected in the droppings, but a degradation product was isolated from them which appeared to be sixteen times as active *in vitro* as pamaquin (Josephson, Taylor, Greenberg & Ray, 1951). These observations all agree in suggesting that in the body pamaquin is metabolized to a more active compound.

In *P. gallinaceum* resistance to pamaquin developed so slowly that, if strains of human malaria react to the drug in a similar way, there is little cause to fear the development of pamaquin-resistance as a result of treatment; nor have resistant strains of human malaria been described up to the present. It must be borne in mind, however, that resistance to this drug developed more readily in *P. knowlesi* than in *P. gallinaceum*, and that species and strains of malaria may vary in their ability to become drug-resistant. With regard to 8-aminoquinolines other than pamaquin, it is a matter of some interest to determine whether these are able to produce drug-resistance with greater facility than pamaquin, and more especially whether they are able to develop resistance to quinine. The degree of resistance to quinine conferred by pamaquin in *P. gallinaceum* is only slight; but a drug which could confer resistance to quinine readily in human malaria would be an undesirable addition to the pharmacopoeia.

SUMMARY

1. A four- to eight-fold increase in resistance to pamaquin has been developed in a strain of *Plasmodium gallinaceum* in chicks.

2. Pamaquin resistance conferred no resistance to proguanil, sulphadiazine, mepacrine, chloroquine or sontochin, but it conferred some resistance to pentaquine and to quinine.

3. An appreciable loss in resistance to pamaquin was observed in the pamaquin-resistant strain after it had been maintained in the absence of the drug, in a patent state of infection, for a period of 6 months.

4. No synergism was observed between pamaquin and quinine when these drugs were tested, in combined doses, upon active infections of *P. gallinaceum*.

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TWO SKIN PARASITES OF *RANA TEMPORARIA: DERMOCYSTIDIUM RANAE* GUYÉNOT & NAVILLE AND *DERMOSPORIDIUM GRANULOSUM* N.SP.

By O. BROZ and M. PRIVORA

From the Department of Parasitology, Charles IV University, Prague

(With Plates II and III and 2 Figures in the Text)

INTRODUCTION

In the skin of *Rana temporaria* L., from the vicinity of Hradec Králové, Czechoslovakia, we found two taxonomically related unicellular parasites. One of them is *Dermocystidium ranae* Guyénot & Naville, the other is a form resembling *Dermosporidium hylae* Carini. Since the former is incompletely known and the latter proved to be a new form, an account is given of their morphology and bionomics.

METHODS OF INVESTIGATION

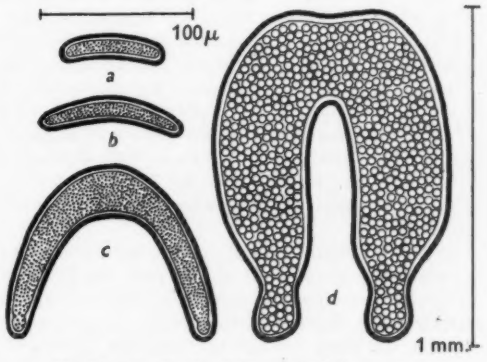
The fresh contents of the cysts were examined microscopically. For histological examination, the material was fixed by one of the following methods: Bouin, Zenker, formol 10%, sublimate-alcohol. It was then embedded in paraffin and sections 9 μ thick were cut. For staining, one of the following methods was used: Masson (haematoxylin-erythrosin-safranine), Mann, Heidenhain, orcein, resorcin-fuchsin, Feulgen. In addition, smears of the cyst contents were stained by Giemsa's method.

I. **Dermocystidium ranae** Guyénot & Naville

This parasite was described by Guyénot & Naville (1922) from the skin of *Rana temporaria* from Switzerland. Remy (1931) found it in France on *R. esculenta*. These authors observed only cysts with mature spores. These cysts are U-shaped with constricted round ends. The interior is a single cavity not divided by septa; it is filled with spherical spores, all in the same stage of development, 8–10 μ in diameter, and each provided with a membrane. In the centre there is a bulky inclusion, while the cytoplasm forms a narrow rim containing the nucleus.

We found *Dermocystidium ranae* in the vicinity of Hradec Králové (Czechoslovakia) only on *Rana temporaria*. A search in other frogs, especially *R. esculenta*, was without results. We found two clear-cut types of cysts, which are easily distinguishable by their localization, shape and contents, and represent two stages of development. Stage I is younger and was to a certain extent described previously by one of us (Broz, 1944). Stage II is the mature cyst with ripe spores described by Guyénot & Naville (1922).

Stage I. Cysts of small dimensions (Text-fig. 1, *a–c*). On the surface of the skin of adult frogs the cysts are not visible to the naked eye so that they have to be looked for on the underside of the skin after this has been removed. Cysts on the belly of young frogs form an exception, as they slightly raise the thin skin in the form of barely visible tubercles.



Text-fig. 1. Cysts of *Dermocystidium ranae*. *a–c*, stage I; *d*, stage II.

The cysts have in general the shape of a bent cylinder, the length and curvature of which are variable and depend on the age of the cyst. We believe that the youngest are the cysts in the shape of a short cylinder with rounded ends, showing only a slight indication of future curvature, and measuring 90 μ in length. In the course of its further development, the cyst increases in length, and also slightly in width, and simultaneously it bends into a V-shape. The two arms of the 'V' form an angle, which is first obtuse and later acute, while the ripe cysts become U-shaped with parallel arms. Each cyst lies in the centre of a tiny granuloma formed by cells of fibroblast and histiocyte type. Local leucocyte infiltrations are not present in the vicinity of this stage. The cyst wall is 2–3 μ thick, staining

intensely with orcein; therefore it has the character of elastic connective tissue fibres. Under higher powers it is seen that this membrane is composed of two layers passing into each other more or less gradually. The internal two-thirds of the membrane are homogeneous and structureless, while the external third is formed by a dense network of relatively thick elastic fibres, which originate from the cytoplasm of the host's fibroblasts as plasmatic fibres. Near the membrane they change their character and pass into the membrane as elastic fibres. In sections stained with orcein the surface of the cyst therefore appears to be hirsute (Pl. II, figs. 1, 3).

The nuclei of the fibroblasts situated nearest to the cyst lie at a certain distance from the membrane, and the cells themselves are orientated in such a way that their longitudinal axis is perpendicular to the surface of the cyst. The membrane and the contents of the cyst seem to be sharply delimited from each other. On the basis of these findings, we are in agreement with Moral (1913) and opposed to the views of most other authors, in regarding the membrane as a product of the cells of the host and not of the parasite. For a more detailed description of the origin of the membrane see Broz (1944). The cyst-membrane remains thinner at both poles, so that the globular constriction characteristic for mature cysts is already indicated. The cyst contents are finely granular. The granules are oxyphile and stain like the inclusions of ripe spores of stage II (red by Mann's method and black with Heidenhain's haematoxylin). In this stage we failed to demonstrate the nuclear substance with Feulgen's reaction.

In the granular contents of several cysts there were many large optically empty vacuoles, between which the granular substance was compressed into narrow strips. The significance of the vacuoles is not clear to us. They are not artifacts, for cysts with vacuoles were found in fresh as well as in fixed material. Jirovec (1939) regularly found similar vacuoles in young stages of *Dermocystidium vejdovskyi*.

Stage II. The cysts, which are visible to the naked eye, have the appearance of an elongated cylinder bent in the form of U, both arms of which are close to each other and almost parallel. Their ends form incompletely constricted globules (Text-fig. 1, *d*). The cyst is densely packed with round spores containing one central, relatively large inclusion-body. This stage was sufficiently described by Guyénot & Naville (1922) to whose account we can only add that, in material fixed with 10% formol, the inclusion stained intensively brown by Lugol's iodine, whereas the above-named authors, who tested the influence of iodine on the living spores, did not find this affinity. Owing to the increasing pressure of the growing cyst, the cells of the granuloma in which the cyst lies are orientated differently from those enclosing stage I; their long axis is not perpendicular to the surface of the cyst, but parallel with it. The elastic fibres composing the external layer of the membrane are not so clearly differentiated, but the membrane still stains intensively with orcein and resorcin-fuchsin. This stage represents the final phase of development of *D. ranae* in the skin of the frog. When the cyst ruptures on the surface of the skin, the spores escape into the surrounding medium (water), but those which remain in the connective tissue of the skin are phagocyted by leucocytes, which now abundantly infiltrate the neighbourhood of the cyst.

The morphological difference of the two developmental stages is correlated with differences in their seasonal occurrence. Stage I was found all the year round in adult frogs, as well as in young ones, whereas stage II occurred only in April in adult, mating frogs. We never found this stage either in summer or in autumn. The incidence of infection in frogs of our area is about 5%.

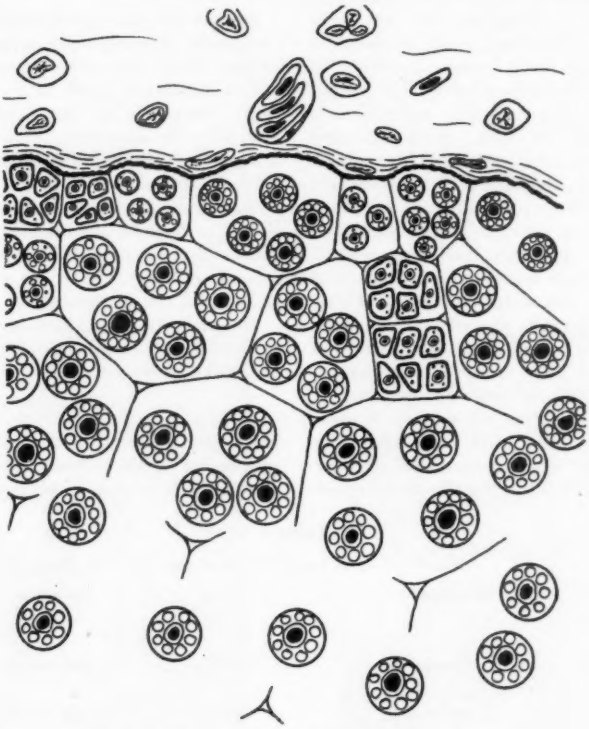
It is not yet possible to give a complete account of the life cycle of this organism. But, on the basis of the differences found in the occurrence and morphology of the two stages, it would appear to proceed as follows. The infection of frogs may take place at any stage of their life after metamorphosis; however, nothing is known about the early stage of the parasite, the portal of entry of the infection, and the method of its transmission. The parasite establishes itself in the subcutaneous connective tissue, where it provokes a local reaction in the form of a small granuloma, with the formation of a membrane around the stage I cyst. In the course of its further development, the cyst grows in length and changes in shape, while its granular contents do not undergo any qualitative changes (Text-fig. 1, *a–c*). The parasite remains at this stage till the next mating of its host. In frogs infected in the first year of their life, this latency lasts 2–3 years, in adult frogs probably only till the following spring. We kept young frogs with a macroscopically detectable infection in the thin skin of the belly for several months in a terrarium without observing any change in the size of the lesion. Subsequent microscopic examination revealed stage I, thus confirming our assumption.

The factors causing the transformation of stage I into stage II, which takes place at the next mating, are not known, but we think that it is connected with hormonal changes in the body of the host. The hormones can act on the parasite either directly or, more probably, indirectly, by producing changes in the skin (oedema of the connective tissue), which stimulate the development of the parasite. Probably the transformation takes place fairly rapidly, as

transitions between stages I and II are rare (they were found only once). As the cyst increases in size, it exercises an upward pressure upon the layers of the skin covering it. When the cyst reaches the surface of the skin, it becomes exposed, owing to ulceration of the attenuated epithelium, and its membrane ruptures, setting the spores free. After emptying of the cyst, the lesion cicatrizes. Sometimes, however, a cyst of stage I does not develop further, dies and becomes calcified. Neither we, nor any other author who has tried, have so far succeeded in artificially infecting healthy frogs with the contents of a cyst.

that it is a new species. The largest number of such lesions in one frog was three, each containing only one cyst.

Histological sections of the skin show a parasitic cyst lying between the skin glands in the stratum spongiosum corii close below the epidermis (i.e. above the stratum compactum). The oedema of the stratum spongiosum, characteristic for the skin of mating frogs, is considerably increased in the immediate neighbourhood of the cyst, producing the hemispherical swelling already mentioned. The epithelium above the cyst becomes thinner, and is later on broken by ulceration, so that after the



Text-fig. 2. *Dermosporidium granulosum* n.sp.

II. **Dermosporidium granulosum** n.sp.

In April 1947 we found on the skin of many *Rana temporaria* elevations of almost regular hemispherical shape, 4–8 mm. in diameter. At the top they had a shallow pit, often with a whitish base (Pl. III, fig. 1). This base, as we later ascertained, was formed by the denuded upper pole of the cyst of a parasite which at first we considered to be an unknown developmental stage of *Dermocystidium ranae*, but the microscopic examination showed

rupture of the wall of the cyst the spores are released on the surface of the skin. In the vicinity of the cyst there is a cellular infiltration of lymphocytes, histiocytes and polymorphonuclear leucocytes. This infiltration is less marked in those cysts that have not yet burst. The shape of the cyst is ovoid or spherical, and it measures about 1 mm. in diameter. Its wall proper is formed by a thin, sometimes barely visible, membrane which stains with resorcin-fuchsin. A thin layer of fibrous tissue orientated parallel to the membrane lies on the outside of this

membrane. The interior of the cyst is divided by thin septa into small chambers. These chambers enclose groups of spores at different stages of development, those in the centre of the cyst containing ripe spores, whereas at the periphery near the membrane are the young stages (sporoblasts) (Text-fig. 2).

The sporoblasts are polygonal cells measuring on the average 7 μ across. They are situated in distinct groups, each of which lies in a separate chamber and consists of cells of the same appearance and size, lying close together and mutually flattened against each other. In the centre of each cell or slightly eccentrically there is a nucleus, in which a distinct membrane and a large nucleolus, but no other structure, can be seen. The presence of thymonucleic acid could not be detected by Feulgen's method in sections fixed with sublimate-alcohol and hydrolysed by N/1-HCl for 4–7 min. The cytoplasm is foam-like with some fine granules. As they develop further, the sporoblasts are transformed into spores. The cells become larger, rounded and finally detach themselves from the groups in which they were held together. The granules in the cytoplasm enlarge and are transformed into inclusion-bodies of characteristic appearance. These are rounded, about 2 μ in diameter, and consist of a membrane and a central body, which sometimes fills almost the whole inclusion. In the young spores there are only a few inclusion-bodies, but later they become more numerous, until they fill the entire cytoplasm.

The ripe spore is a spherical cell with an average diameter of 10 μ. It has a thin membrane, distinguishable especially when stained with Heidenhain's haematoxylin. Thus it differs from the sporoblast in larger size, spherical shape and in the presence of numerous inclusions in the cytoplasm. In a fresh preparation, the spores appear under the microscope as globules provided with a distinct membrane and filled with numerous large granules (Pl. III, fig. 3).

The parasite occurred rather irregularly. Though we have studied the skin parasites of frogs since 1942, we found it only in 1947 and 1949. Like *Dermocystidium ranae*, it occurred only during the mating time of *Rana temporaria*, but a little later than in that parasite. In 1947 it affected about 30 % of the frogs, but in 1949 we found it only on two individuals.

Because of the considerable resemblance of this parasite to *Dermosporidium hylae* Carini we propose to name it *D. granulosum* n.sp.

D. hylae Carini (1940) is a parasite of *Hyla rubra* from Brazil. It produces spherical-shaped cysts, 400–500 μ in diameter, situated close under the epidermis. They contain spherical or slightly ovoid spores, measuring 8–10 μ. In the cytoplasm of the spores there are black staining granules of various size. Carini does not describe the structure of the spores in greater detail, and does not mention the nucleus at all, probably on account of the similar staining of the inclusions and the nucleus.

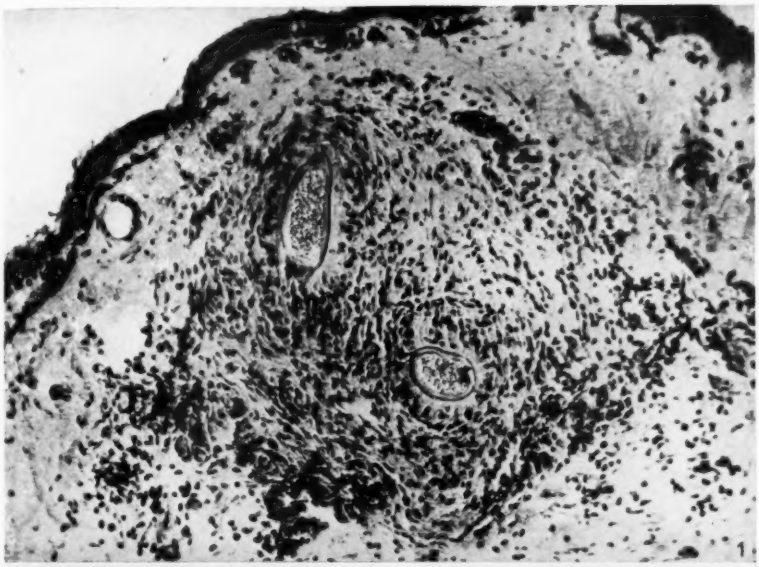
Recently Weiser (1949) reported the occurrence of a similar parasite, *Dermosporidium truttae*, from the gills of *Trutta fario*.

TAXONOMY

Both species described in this paper are related to a number of parasites occurring, on the one hand, in the skin of various amphibians and, on the other

Table 1

Name	Host	Localization of the cyst	Shape	Spore inclusions
(1) *Dermocystidium bronchiale* Léger, 1914	*Trutta fario*	Gills	Spherical	One large central
(2) *D. salmonis* Davis, 1947	Salmon	Gills	Spherical	One large central
(3) *D. vejdovskyi* Jirovec, 1939	*Esox lucius*	Gills	Spherical	One large central
(4) *D. pusula* Pérez, 1907	Different species of *Triton*	Subcutaneous	Spherical	One large central
(5) *D. ranae* Guyénot & Naville, 1922	*Rana temporaria, R. esculenta*?	Subcutaneous	U-shaped	One large central
(6) *Dermosporidium hylae* Carini, 1940	*Hyla rubra*	Subcutaneous (stratum spongiosum corii)	Spherical	Numerous
(7) *D. granulosum* n.sp.	*Rana temporaria*	Subcutaneous (stratum spongiosum corii)	Spherical	Numerous
(8) *D. truttae* Weiser, 1949	*Trutta fario*	Gills	Spherical	Numerous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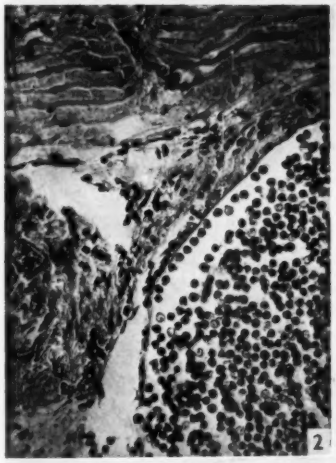
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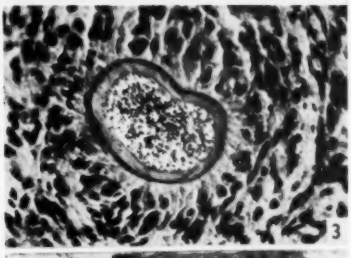
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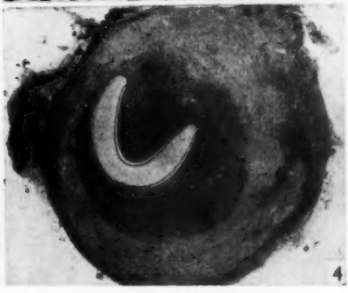
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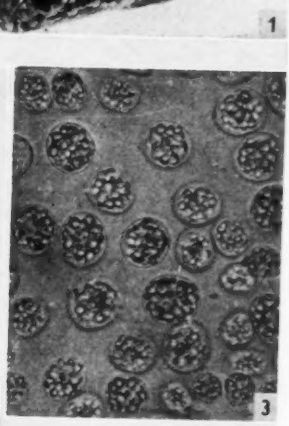

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hand, in the gills of some fish. Two genera were established for them: *Dermocystidium* Perez and *Dermosporidium* Carini. All these organisms produce cysts containing spores with inclusions. The species differ from each other chiefly in the shape and localization of the cysts, in the structure of the spores, as well as in the type of host. The main differential characters are given in Table 1.

It is not yet clear whether these organisms are Protozoa or lower parasitic Fungi. Most authors place them among the Haplosporidia, a group which is not yet accurately defined and classified.

Here it may be noted that the parasites of newts (*Triton* spp.), *Dermomycoides armoriacus* Poisson and *D. beccarii* Granata, were originally attributed to the genus *Dermocystidium* (Beauchamp, 1914). However, since their spores differ from those of both *Dermocystidium* and *Dermosporidium* in the presence of flagella and in the absence of inclusions, Poisson (1937) places them among the lower Fungi of the family Chytridinae. Another species of *Dermocystidium*, *D. daphniae* Jirovec, is now known as *Lymphocystidium daphniae*.

SUMMARY

In the skin of *Rana temporaria* from Czechoslovakia two unicellular parasites have been found: *Dermocystidium ranae* Guyénot & Naville and *Dermosporidium granulosum* n.sp.

Dermocystidium ranae. Two stages were found, one of which is new. An account is given of the morphology, development and occurrence of both stages, including details which have hitherto not been recorded.

Dermosporidium granulosum n.sp. This species is represented by cysts situated in the stratum spongiosum corii. They are divided by septa into small chambers containing groups of spherical spores with a central nucleus and with numerous small cytoplasmic inclusions. Within the cyst, the sporoblasts occupy the periphery, while the ripe spores are in the centre.

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EXPLANATION OF PLATES II AND III

PLATE II

Fig. 1. *Dermocystidium ranae*. The small granuloma in the skin of *Rana temporaria*, formed by fibroblast- and histiocyte-type cells and containing V-shaped cyst of stage I. Both arms are in section.

Fig. 2. *D. ranae*. The margin of the mature cyst with ripe spores.

Fig. 3. *D. ranae*. Section through stage I cyst, containing fine granules.

Fig. 4. *D. ranae*. Granuloma with young cyst of stage I, in fresh preparation.

PLATE III

Fig. 1. Hemispherical elevation on the skin of *Rana temporaria* containing a cyst of *Dermosporidium granulosum* n.sp. (indicated by arrow).

Fig. 2. *Dermocystidium ranae*. Cysts on the skin of *Rana temporaria* (indicated by arrows).

Fig. 3. *Dermosporidium granulosum* n.sp. Living spores.

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REMARKS ON THEILERIOSES AND *THEILERIA*

By E. DSCHUNKOWSKY

The group of diseases discovered at the beginning of this century, and known as theilerioses, is still very much of a riddle, and much regarding it is still open to further investigation. In judging of the importance of such investigation it should be borne in mind that within the enzootic areas improvement in local cattle breeding is impossible and that, in addition, it is very difficult to apply to this group measures that are taken against other epizootic diseases.

In respect to the causative organism the zoological position of parasites of the family Theileriidae must be considered as still provisional. For instance, Cowdry & Ham (1930), basing their views on a similarity in development, place *Theileria* with *Plasmodium*. On the other hand, Reichenow (1932), 'n'arrive pas aux mêmes conclusions. Il ne voit pas les formes mâles et femelles, les forms vermiculaires de Cowdry et Ham ou les grandes formes irrégulières provenant du noyau (sporonts), et suppose que les deux auteurs ont pu rencontrer une infection mixte à *Babesia*' (Curasson, 1943, p. 270). This author finds an affinity between *Theileria* and *Piroplasma*. Conclusions based on the common transmission of *Theileria* and *Piroplasma* by ticks must, however, be accepted with caution, since ticks may well be simultaneously vectors of quite unrelated forms of blood parasites.

As regards the species to be recognized in the Theileriidae, workers who have studied the family have as yet come to no definite opinion. Some consider there to be four well-recognized 'good species' viz. *Theileria parva*, *T. annulata*, *T. dispar* and *T. mutans*. Others consider these to be forms of a single species. No definite conclusion has been reached as to what are the criteria on which 'good species' in this case can be founded. Are the parasites to be classed on the degree of their virulence, or according to the type of vector tick? If the degree of virulence is to be taken as basis then the validity of many forms will become uncertain, and Du Toit (1930) will be correct in accepting only the four above-mentioned forms as good species. If the cautious view of Du Toit is not accepted, then the question of 'good species' is still open.

Lately some observers have considered *T. dispar* to be a synonym of *T. annulata* (Delpy, 1949; Witenberg, 1947), and Sergent, Donatien, Parrot & Lestoquard (1945), in Algiers, write as follows: 'Nous avons montré, par l'épreuve de la prémunition croisée, que l'agent de la theilériose bovine du Moyen Orient (Palestine, Iran) est spécifiquement identique à *T. dispar*.' Sergent, Donatien, Parrot, Lestoquard, Plantureux & Rougebief (1924) also say of this species: 'Il apparait donc aujourd'hui comme un synonym de *T. annulata* (Dschunkowsky et Luhs, 1904) observé en Transcaucasie.' But later, in a discussion between the present author (1948*b*) and Sergent, Donatien & Parrot (1948), the French scientists changed their point of view and considered that *T. dispar* and *T. annulata* were both good species. This new opinion is worthy of consideration, since there is no doubt that it may serve as a starting-point for further contributions to the question of species of *Theileria*.

It has already been stated that the identification of species of *Theileria* based on the type of transmitting tick cannot be regarded as reliable. It is sufficient to point out that besides *Rhipicephalus appendiculatus* and *Haemaphysalis bispinosa* as vectors of *T. mutans*, the part played by such representatives of the original genus *Boophilus* as *B. annulatus* and *B. microplus* in Australia is still far from being clear, though it must be pointed out that according to present views representatives of the genus *Boophilus* are not considered to be vectors of *Theileria*.

Then again the important question of distribution of these diseases is still imperfectly known. In the report of the author (Dschunkowsky, 1948*a*), submitted to the French Academy of Science by Prof. Roubaud, the northern limit of distribution of the theilerioses in Europe and in the Near and Far East is given as 42–43° northern latitude. More recently Yakimoff & Dekhtereff (1930) have recorded *T. sergentii* in the far east of Asiatic Russia in still more northerly latitude. Thus the theilerioses are the most widespread diseases of our domestic animals.

At the present time the theilerioses are known in Europe, Africa, Asia and Australia. In Australia they were first observed by Dodd in 1912 at Yeerongpilly, Brisbane. According to Legg (1935):* 'He noted their appearance in blood smears of animals which had been inoculated for "tick-fever" and considered them identical with the Theiler organism, but his point of view was not accepted. Pound refers, in his annual reports, to the rod and ring forms of *P. bigeminum* in Queensland cattle. There is no doubt from his description that he was confusing *T. mutans* with *P. bigeminum*.'

We as yet know nothing of vast unexplored regions

* In his letter of 18 August 1949 to the author Dr Legg says that in Australia there is only one species of *Theileria*, i.e. *T. mutans*. It is very widespread throughout the tick-infested areas (*Boophilus microplus*) of Australia and has never been known to cause any harm.

of the Old World serving as pasture for millions of local cattle. It is quite possible that in any of these are regions where newly imported cattle may become infected with *Theileria*, whereas locally bred animals do not show theileriosis unless they are kept under particularly hard conditions. The author, for example, discovered in Transcaucasia the existence of a new disease (theileriosis) exhibited only in connexion with vaccination by a combined method against cattle plague in 1902. The difficulty in identifying the species of *Theileria* already referred to, when all the cattle of an area are in a condition of premunition in respect to theileriosis, as well as piroplasmosis and anaplasmosis, explains why so little is known of the forms of *Theileria* in different countries. This may also be the explanation why the theilerioses are not yet known in the New World, since it is strange that these diseases are known, neither in North, nor, especially surprisingly, in South America, and especially when, according to the data of the Bureau of Entomology and Plant Quarantine, there exist in these parts of the world species of tick that are elsewhere vectors of various blood parasites including the theilerias.

In this article we do not propose to review all the many contributions that have been made to knowledge of the theilerioses,* but to confine our remarks to discussion of the important question of the development of *Theileria* in the circulating blood of sick animals.

The characteristic feature of the genus *Theileria* is the development of these parasites in the leucocytes and in the cells of the endothelial tissues of tick-infested animals. For example, until Koch's bodies had been discovered in the course of development of *T. mutans* these parasites were classed as *Piroplasma*, and later a special genus *Gonderia* was created for them. There is, however, also the question of development in the erythrocytes of the circulating blood to be considered. If the view of Du Toit is accepted, the development of *Theileria* by means of Koch's bodies is observed as a rule only in the exceptionally virulent *T. parva*. In *T. annulata* and *T. dispar* 'blue bodies' appear only in severe infections, and in the least virulent of the forms (*T. mutans*) they are found only in exceptional cases. Thus in the less virulent forms of the parasite development takes place chiefly in the erythrocytes of the circulating blood, where also are formed the sexual stages destined to develop further in the tick. The occurrence of such development independently of the part played by the 'blue bodies' is also indicated by the comparative readiness with which susceptible animals are infected by blood containing *T. annulata*, *T. dispar* and *T. mutans* parasites.

All this leaves the connexion between the two forms of development still very uncertain. We do not know whether the infection from the tick is of two distinct species, or whether of the young parasites injected some are intended for development in the endothelial system, while others are for development in the erythrocytes of the circulating blood. If they are of the same species, why in some cases do they develop exclusively in the cells of the lymphatic system as in *T. parva*, while in other cases they develop both in this situation and in the circulating blood (*T. annulata* and *T. dispar*), and in a third case development is confined almost exclusively to the circulating blood (*T. mutans*)?

As regards the actual form which development in the erythrocytes takes authors give only its general features. Thus Curasson (1943), when summarizing all that is known regarding the development of *T. mutans*, says briefly: 'L'existence de corps en grenade indique que le mode d'évolution est le même que pour les autres theileries.' But as already mentioned, in the case of *T. mutans* development occurs almost exclusively in erythrocytes of the circulating blood and the formation of blue bodies ('corps en grenade') in the cells of the lymphatic system is observed only as a rare exception. Moreover, there is no indication of development of parasites in the blood in the case of *T. dispar*. Sergent *et al.* (1945), speak of *T. dispar* as a purely tissue parasite, and the same is indicated in the scheme of development these authors give for the life cycle of the parasite in the mammalian host and in the tick. Speaking of *T. annulata*, they state that they have never seen a transition from ring forms into the anaplasmatic form and vice versa. There are, however, already indications in the literature as to the possibility of development of *Theileria* in the circulating blood and of the presence of anaplasmatic forms (a feature which we have mentioned as early as 1927). Yakimoff & Dekthereff (1930), speaking of *T. sergenti* of the Far East say: 'Elle diffère de *T. parva* par les caractères suivants: la masse chromatique est parfois divisée en 2 ou 3 parties' (Curasson, 1943). Delpy (1949), working in Iran (Persia) under conditions similar to those in Transcaucasia, writes: 'Nous avons montré en 1937 que dans le sang vivant, la forme des éléments erythrocitaires varie continuellement, ce qui s'explique selon nous, par le existence d'un processus de division binaire. Il est donc normal que la même *Theileria* soit vue et décrite sous les aspects différents par des auteurs qui n'étudient qu'un nombre limité de frottis colorés. Les seules caractéristiques constantes de l'espèce sont que les formes renflées sont toujours plus nombreuses que les formes allongées, et que la chromatine des formes allongées est toujours punctiforme ou sphérique.'

* We have already done this in our monograph on the theilerioses which is being published by the firm of Gustav Fisher (Jena). Unfortunately, the twenty-seven coloured plates to accompany this, together with the negatives, have been destroyed by bomb damage and will be impossible to replace.

It seems desirable, therefore, that we should give a full description of development in the erythrocyte, as has been given by me in my work already referred to.

In the chromatin mass of a full-grown parasite of *T. annulata*, which lies in the form of a crescent on the periphery of the parasite, the following changes take place. Parts of the mass shrink, whilst there appear two, three or four protuberances (future chromatin masses). These strange-looking excrescences, at first seen against a background of chromatin substance, gradually change into separate masses which for a time remain in connexion with each other in various forms depending on the chromatin arrangement of particular parasites. Finally they separate, forming two, three or four separate chromatin masses. At the same time the cytoplasm diminishes, becoming reduced in amount about the newly formed nuclear masses, and finally disappears entirely leaving a cross-like arrangement of dot-like parasites such as have been described by many authors. Sometimes, however, groups of two or three dot-like parasites only are seen. According to certain authors this separation into four parts is characteristic for the family Theileriidae.

In these newly formed dot-like parasites the cytoplasm after becoming reduced is present only in very insignificant amount. A study of the structure of such forms is difficult, and successful only under good illumination and in perfectly stained preparations. The following is the disposition of the cytoplasm in these dot-like parasites. In the body of such an 'anaplasm', consisting apparently of a single circular chromatin mass, is a sector-like fissure filled with bluish stained material, undoubtedly cytoplasm. In smears we were able to note parasites with increasing amounts of such cytoplasm, the sector appearing in some parasites to be only a small fissure, whilst in others it was expanded until it occupied one-sixth or even one-quarter of the circle. With further development of the cytoplasm the parasites lose their dot-like appearance and form rings rich in chromatin, the latter being in the form of larger or smaller bodies pushed to the periphery. At first the cytoplasm and chromatin are equal in amount, but as the parasite matures the cytoplasm increases considerably in relation to the chromatin, which eventually becomes disposed crescentically on the periphery of the parasite, which now has the form of a typical small ring of *T. annulata*.

While studying these changes we have observed certain forms to which we ascribe the significance of gametes. In these the chromatin is arranged in crescentic form, but later gradually takes on a rounded shape. This change commences by the inner border of the crescent becoming straighter and then gradually protuberant until finally the mass is quite round. In some cases the cytoplasm gradually disappears leaving an anaplasmatic body (microgamete). A similar process is seen in those forms which we consider to be macrogametes. These last lose their cytoplasm and the chromatin acquires a round form constituting an anaplasmatic body. The actual transformation in such a process may vary. Thus for *T. annulata* the anaplasmatic and ring-shaped forms have a different significance. In certain cases they may be a stage in agametogonic development of the parasite, while in others they may be in the nature of gametes.

However, the significance of the dot-like forms is not limited to the process described above. Sometimes appearances are seen which indicate the multiplication of the anaplasmatic forms into groups of as many as six parasites which have not separated. This curious appearance might be explained by the fact that division has occurred rapidly before the chromatin has become disposed in its usual place and form in the parasite. Such bodies may be of various dimensions (0·85–1·5 μ) as is also mentioned by Sergent. They appear to be the result of a continuation of the process of division as described above when the cytoplasm disappears early. The capacity of losing cytoplasm easily appears to represent a general characteristic feature of these parasites.

These forms in the development of *Theileria* (*T. annulata*) in the circulating blood have already been figured by me (Dschunkowsky, 1927). The figures given by Delpy (Curasson, 1943, table XXVII) fully correspond to these. In his figures 1, 2, 3, 11 and 12 are the anaplasmatic forms after separation of their chromatin mass into two and four pre-division parasites. They correspond to my figures 9 and 28. His figure 3, showing separation into 2, 3 and 4 daughter cells, corresponds to my nos. 7, 8, 23 and 24. Thus the transformation of the chromatin mass characteristic of the cycle of development as described by me in the circulating blood is also confirmed by Delpy in his observations. In addition, my figures relating to the formation of sexual forms and showing bodies with round chromatin masses correspond to those of Delpy.

As to the formation of ring-shaped parasites from anaplasmatic forms, a conclusion can be arrived at by comparing the proportions of chromatin and cytoplasm in the different stages shown in my figures. The same can be said regarding the diagram showing the development of *T. dispar* in the circulating blood given by Sergent *et al.* (1945, p. 443). Among these authors' figures can be seen the same forms as are given in my scheme of development for *T. annulata*, viz. division of the chromatin into 2, 3 and 4 parts, and anaplasmatic forms losing their cytoplasm. Thus in their figure 194 not only are gametocytes shown in the circulating blood, but also forms of agametogonic nature.

We have checked once more notes in our possession relating to charts made at an earlier date. They leave no doubt as to the fact that such changes as

we have described actually take place in *T. annulata* in the circulating blood. On the temperature chart of an animal sick with this disease we recorded on 24 July 1904 as follows: 'A change of point-shaped forms into ring-shaped parasites, the number of which on 30 July 1904 has reached 10 %, has been observed in the blood of ox no. 267 with temperature 41° C.' In this case it was a matter of transformation of anaplasmatic forms into ring-shaped forms with a gradual increase of cytoplasm in the fissure in the chromatin mass of the anaplasmatic body, observable, as mentioned before, only under good illumination and perfect staining. The extract refers to *T. annulata* and is sufficiently convincing as to formation of ring-shaped forms from anaplasmatic forms in the development of this parasite in the circulating blood of a sick animal. From the drawings by Sergent *et al.* (1945) to which we have referred, we can say the same regarding changes in *T. dispar* in the circulating blood.

Unfortunately, scientists working in different laboratories with local material can with difficulty co-ordinate results. This, however, could be done by a special commission of international character working in different areas where theileriasis manifests itself. With such a systematic inquiry it should be possible to co-ordinate the at present conflicting results which have accrued since the discovery of this still little understood, but economically very important, parasite discovered half a century ago.

SUMMARY

1. Areas in which theileriasis occurs are characterized by conditions in which the native cattle do not show clinical manifestations of the disease, but introduced cattle suffer from an epizootic manifesting itself seasonally with varied intensity.

2. The degree of virulence exhibited and the species of tick acting as vectors have only an indirect significance as an indication of species of the parasite.

3. The facts relating to the development of *Theileria* in the tissues of infected animals and relation of this to developmental stages in the circulating blood are still far from clear.

4. The development cycle of *Theileria* in the red cells of the circulating blood of sick animals is described, and proof given that this is characterized by the formation of anaplasmatic forms developing into ring-shaped forms in *T. annulata* and *T. dispar*, and probably in all forms except, possibly, *T. parva*.

5. It is very desirable from the point of view of world economy that a special commission to study theileriasis in the different areas in which it occurs should be established on an international basis.

In the course of writing this paper I have been accommodated in the laboratory of Prof. Roubaud at the Pasteur Institute, and have made use of the library facilities of this establishment. I wish to express my keen gratitude to the Direction of the Pasteur Institute of Paris for having accorded me this hospitality and particularly to Prof. Roubaud for the kind reception he gave me during the time I was working in his laboratory. I also wish to thank Sir Rickard Christophers for going over the English text of may paper. I thank him heartily for this friendly service and I also wish to extend my thanks to Dr Hoare for his advice and suggestions.

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A SPOROZOAN BLOOD PARASITE OF *ARCHIULUS MORELETI* (DIPLOPODA)

By ALFRED J. GIBBS

Honorary Research Assistant in the Department of Zoology, University of Cape Town

(With 13 Figures in the Text)

The blood parasite described in this paper occurs in the diplopod *Archiulus moreleti* (the common garden milliped) which is too well known to warrant any description. The incidence of the infection fluctuates considerably, varying from almost 50% to about 1% in the same locality within the course of a few months. There is apparently no seasonal rhythm of incidence.

The blood cells of the host are feebly amoeboid and, when rounded, measure about 9μ across. The nucleus is usually eccentrically situated and occupies about half the diameter of the cell. When dry-fixed and stained, the nucleus appears as a closely packed mass of pink-staining granules, but after wet-fixation in Schaudinn's solution and staining in Ehrlich's haematoxylin the nucleus reveals a vesicular structure, with a distinct nucleolus. Fig. 1 shows the blood cell dry-fixed and stained, while Fig. 2 depicts the same cell after wet-fixation and staining. It has been found that the best method of obtaining blood for examination is to remove one of the antennae during life, when a sufficient flow of pure blood can be obtained from the wound, but a small dorsal incision, made between segments, will result in a more copious flow.

ASEXUAL PHASE

The method of transmission of the parasite from host to host has not been experimentally proved, but it appears certain that infection follows the ingestion of one or more sporocysts (Figs. 11–13), which will be described later. The sporocyst contains one sporozoite which, presumably, is liberated in the digestive tract and later enters the blood stream. Probably it passes through the gut wall and into the haemocoele. Here it enters the cytoplasm of a blood cell and becomes rounded; it then commences multiplication by binary fission (Fig. 3). As division continues the cytoplasm of the host cell increases in bulk in order to accommodate the organisms, while the nucleus, although not distorted, is pushed to the extreme border of the cell. The maximum number of parasites found in any one blood cell is twenty, which at that stage averages some 20μ across (Fig. 4). The intracellular parasites measure about 2·2μ in diameter; the nuclei are eccentrically situated and exhibit a central karyosome when wet-fixed or examined in the living state. Owing to the extreme smallness of the organism the nuclear division cannot be described.

When the blood cell has become heavily parasitized, its cytoplasm appears to disintegrate and the contained organisms are liberated. They then enter fresh blood cells and the cycle is repeated. It is sometimes difficult to find an uninfected cell. While this phase is proceeding the nuclei of previously parasitized blood cells can be found throughout the blood stream in progressively increasing numbers.

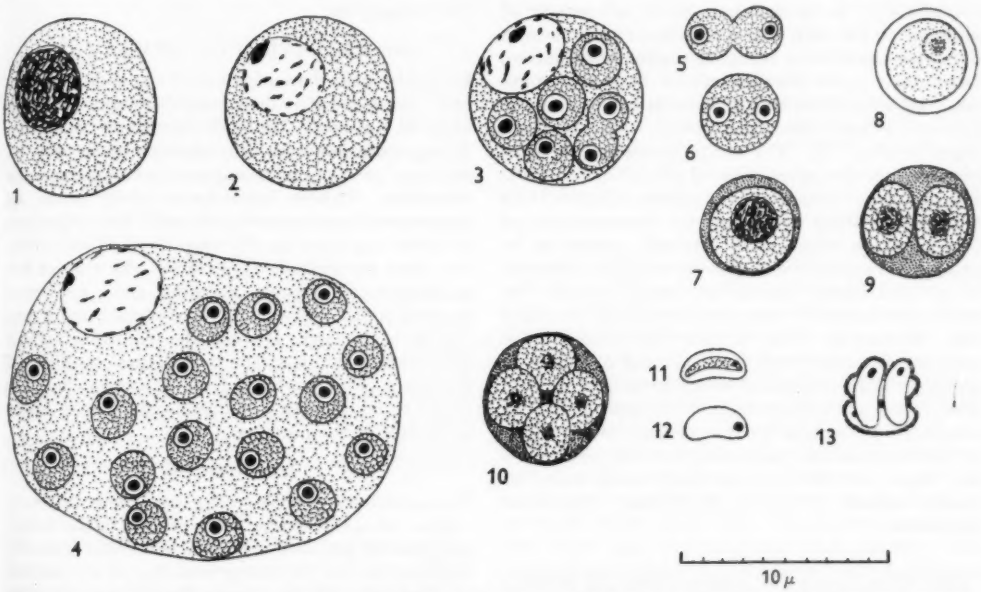
SEXUAL PHASE

The sexual phase usually follows heavy parasitization by the previously described forms, but instances have been noted where, although the infection was so slight as to need careful examination for its detection, the host cells have been found to harbour sexual forms and mature sporocysts. No explanation is offered as to the reason for this, apparently, premature development.

When the parasites enter upon the sexual phase they no longer become free in the blood stream but remain, instead, within the now feebly staining, distended cytoplasm of the host cell. Within each cell approximately the same number of parasites are produced by binary fission, giving rise to gametes, which are morphologically indistinguishable from the earlier asexual forms. The gametes become associated in pairs (Fig. 5) within the cell; cytoplasmic fusion takes place and a spherical, binucleate body, measuring about 5μ across results (Fig. 6). Later the nuclei fuse and the resultant synkaryon, which is situated at one side of the body and is vesicular in structure, has a heavy, central karyosome, while the zygote itself becomes surrounded by a border of pink-staining material which is visible only after treatment by Leishman's method (Fig. 7). In fresh preparations it is clearly seen that the zygote is surrounded by a delicate protective membrane (Fig. 8), which is not visible after fixation, but it is evident that the intervening space between the periphery of the zygote and the membrane contains a strongly staining substance.

The fusion of the gametes has been studied in the living state in saline. It is necessary, in order to observe this phenomenon, that associating gametes should be detected as quickly as possible after removal from the host, and it is also necessary that they should be free in the saline, as it is not possible to study accurately the agglomerated forms within the blood cell. In the course of preparation of the material on the slide, numbers of gametes are liberated, but it is not always easy to locate a pair which have just become applied to each other. The opposite ends. The body soon becomes quite spherical. Normally at this stage the zygote becomes invested in the membrane, but owing to the artificial conditions in which the organism was studied this did not occur, and the zygote soon disintegrated.

The first zygotic division results in the formation of two ovoid bodies within the membrane, which increases somewhat in diameter. When dry-fixed and stained, the pink-staining material is found to fill all parts of the oocyst not occupied by the



Figs. 1–13. Sporozoan parasite of milliped. Figs. 1, 2, normal blood cells (stained after dry- and wet-fixation respectively); Fig. 3, blood cell harbouring parasites produced by repeated binary fission (one dividing form); Fig. 4, blood cell with numerous parasites; Fig. 5, pairing of gametes; Fig. 6, fusion (copulation) of gametes; Fig. 7, zygote showing stained border (Leishman's stain); Fig. 8, zygote showing oocyst membrane (fresh preparation); Fig. 9, oocyst with two sporoblasts; Fig. 10, oocyst (mature) with four sporoblasts; Fig. 11, sporocyst with single sporozoite; Fig. 12, sporocyst as seen after dry-fixation; Fig. 13, group of four sporocysts surrounded by oocyst membrane.

time taken to complete cytoplasmic fusion is between 1 and 4 min., depending, probably, upon the length of time during which the pair had been associated previously. When first attached, the gametes are spherical and the point of contact is necessarily a very small one. The eccentric, vesicular nuclei are clearly visible. Very soon the margin of both partners becomes flattened at the point of contact, this process continuing until each is roughly hemi-spherical in form. Later the margin becomes less apparent along the line of contact until there is, apparently, only one, somewhat elongate, body in which the nuclei are situated near the enclosed cells (Fig. 9). When wet-fixed, the nuclei of these cells are central, vesicular in structure and possess central karyosomes. A further division results in the formation of four ovoid or round sporoblasts, each with a centrally situated nucleus. As in the preceding stage, the space between these sporoblasts, and between the sporoblasts and the enveloping membrane, is filled with pink material after staining by Leishman's method (Fig. 10). Later, each sporoblast becomes elongated and finally banana-shaped. It then becomes invested in a cyst wall (sporocyst), measuring about 3μ long and $1{\cdot}5\mu$ across, and is transformed into a single

sporozoite, in which the nucleus is located near one extremity (Fig. 11). After dry-fixation and staining the sporocyst exhibits a remarkably brilliant, round red body near one extremity, but otherwise there is little detail to be made out (Fig. 12). It is doubtful whether this body is the nucleus of the enclosed sporozoite; it is more probable that it marks the point of exit of the contained sporozoite, and is in the nature of an absorbable or dissolvable 'plug'.

The arrangement of the four sporocysts within the membrane is nearly always the same; two lie together and parallel in the upper plane, while beneath them lie another pair, which are usually at an angle to the first. The membrane can be seen in living material but is not visible after wet-fixation. Leishman's stain reveals only a border of pink material which follows the uneven contour of the sporocysts and also, apparently, cements them together (Fig. 13). The entire development takes place within the infected blood cell, although when sporogony is completed there is little evidence of its cytoplasm, which is replaced by the many groups of sporocysts which have probably grown at its expense. There is evidence, however, of the existence of an ectoplasmic membrane which invests the sporocysts together with the nucleus of the host cell. Frequently three or more parasitized blood cells become fused together, forming a bundle of sporocysts, the number of which is far greater than that found in a single cell, but these cases can always be recognized by the presence of a number of blood-cell nuclei. Generally, however, even when the blood cells lie in a compact mass, they are clearly marked off by the enveloping ectoplasmic membrane.

DISTRIBUTION OF PARASITES IN HOST

During the asexual phase of rapid multiplication the parasites are found in the circulating blood in numbers varying according to the duration of the infection. Later, all stages of the sexual forms can be found, and finally the limbs and antennae of the milliped become packed with sporocysts. If one of the antennae is removed and examined in saline under a cover-glass, pressure will cause the organisms to pour out like dried peas out of a paper bag. Every leg, likewise, contains large numbers of them. However, in some millipeds, the limbs and antennae contain very few parasites, although dissection reveals in them a heavy parasitization of the haemocoele.

During the later stages of sporogony the ectoplasmic pellicle of the parasitized blood cells probably develops a certain stickiness, causing them to adhere together in huge masses. At the same time, healthy blood cells apparently make an effort to reduce the number of parasites, as ingested forms can frequently be found in phagocytes. They usually consist of isolated groups of four sporocysts which have become detached from the mass of host cells. A repetition of the asexual stage has never been observed in the phagocytes.

DISSEMINATION OF SPOROCYSTS

Probably mass dissemination of the sporocysts can only take place after death and disintegration of the body of the host. Millipeds have been found in a dying condition with only stumps of the legs remaining, owing to what appears to be a gangrenous condition. Others have been observed in an apparently healthy condition with the extremities of a few legs missing, the stumps of which exhibit the same gangrenous appearance. In view of this occurrence it appears possible that sporocysts located in the limbs might be disseminated during the life of the host. It should be pointed out that it is not considered that the pathological condition of the limbs is due to the infection, since it has been found to occur among millipeds which were shown to be free of the parasite.

DISCUSSION

The systematic position of the parasite is extremely vague. It would appear to be related to the Schizogregarinida, but its inclusion in this order cannot be justified for the following reasons: (*a*) the asexual multiplication is by binary fission instead of by schizogony; (*b*) the active gregarine or vermicule stage is entirely absent; (*c*) although sexual association takes place between like gametes, as is usual among the gregarines, the resultant zygote forms four sporozoites, each of which is enclosed in a separate cyst-wall and is almost certainly independently resistant to desiccation during external exposure, whereas the schizogregarine zygote produces one sporocyst which contains several otherwise unprotected sporozoites.

In view of these facts, this organism can only be placed among *Sporozoa incertae sedis*.

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THE ECOLOGY OF IMMATURE PHASES OF TRICHOSTRONGYLE NEMATODES

IV. LARVAL POPULATIONS ON LOWLAND PASTURES

By H. D. CROFTON, B.Sc., Ph.D., *Department of Zoology, University of Bristol*

(With 5 Figures in the Text)

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I. INTRODUCTION

In a previous paper (Crofton, 1949) the results of examinations of hill pastures are recorded and discussed. While some of the conclusions drawn from this work may apply to lowland pastures, the special problems of the latter need separate study. The work described below is preliminary to a more extensive and precise investigation of the problems relating to parasite populations on lowland farms.

II. METHODS

The method of estimation of larvae on a pasture was the same as in the previous paper (Crofton, 1949), that is, a modification of the method described by Taylor (1939).

III. DISTRIBUTION OF LARVAE ON PASTURES

When grass types are evenly distributed over a field, so that all parts are equally palatable to the grazing animal, then uniform grazing tends to occur if all parts are equally exposed to climatic changes. Lack of uniformity in the sward is reflected by the varying degrees to which different parts of the pasture are grazed, and this lack of uniformity is itself further accentuated unless grazing is carefully controlled. In general, lowland pastures are more evenly grazed than upland pastures, but considerable variation occurs. Mixed stocking may give rise to uneven swards where management is poor, or where special soil conditions occur, but usually judicious grazing with sheep and cattle produces a good uniform grazing, or preserves it. The introduction of horses into a field usually results in a loss of uniformity. When a field is not evenly grazed the numbers of infective larvae in different parts of the pasture must vary considerably because first, the distribution of faecal pellets must bear some relation to the grazing, and secondly differences in microclimate produced by uneven herbage will be reflected by the different times of survival of larvae.

Uniform grazing, however, may be expected to give rise to a random distribution of clumps of faeces, and therefore to a random distribution of groups of worm eggs. Since the larvae developing from these eggs have been shown to migrate only small distances laterally (Augustine, 1923; Mönnig, 1930; Lucker, 1938; Furman, 1944; Dinaburg, 1944), then one would neither expect the larvae themselves to be distributed at random, nor the frequency distribution of larvae in random samples of grass to fit a Poisson curve. Neyman (1939), however, suggested formulae to fit types of distributions where organisms or cells are arranged in clumps at random. Of these, only the form known as Neyman Contagious Distribution Type A will be considered, the refinements of his Types B and C being not only laborious to manipulate but unnecessary in this type of problem.

In N.C.D. Type A, with two parameters, the probability that the number of larvae (X) occurring on a given unit area will be given by

$$P\{X=n\}=e^{-m_1}\frac{m_2{}^n}{n!}\frac{d^n}{du^n}e^{m_1e^u-m_2}\Big|_{u=0}.$$

This in the recurrent form gives (see Beall, 1940)

$$P=\{X=n+1\}=\frac{m_1m_2e^{-m_2}}{n+1}\sum_{k=0}^{n}\frac{m_2{}^k}{k!}P\{X=n-k\},$$

where m_1, m_2 are parameters (see Neyman, 1939; Beall, 1940).

To test the goodness of fit of N.C.D. Type A for the distribution of larvae on pastures, three lowland pastures were examined. The pastures were chosen because they were known to be infested and because the sward appeared to be evenly grazed.

Seventy-five samples were taken at random from the central, most evenly grazed part of each. Each sample, of approximately fifty blades of grass which had been growing adjacent to one another, was washed. The washings were centrifuged and examined for larvae. The results are given in Table 1 which also includes the theoretical frequencies expected from Poisson Distribution and the frequencies calculated from the N.C.D. Type A formula.

Table 1. *Frequency distribution of larvae in samples*

	Field A. Frequency			Field B. Frequency			Field C. Frequency		
		Calculated			Calculated			Calculated	
No. of larvae	Observed	N.C.D.	Poisson	Observed	N.C.D.	Poisson	Observed	N.C.D.	Poisson
0	44	43·537	18·74	56	56·820	36	45	46·79	24·147
1	5	6·102	25·98	3	3·769	26·25	10	6·49	27·366
2	8	7·419	18·02	7	4·653	9·75	6	7·28	15·507
3	6	6·342	8·27	5	3·955	2·25	5	5·77	5·858
4	5	4·439	2·87	2	2·700	0·75	3	3·77	1·659
5	3	2·824	0·79	0	1·682		4	2·24	0·376
6	2	1·576	0·18	1	1·013		0	1·16	0·071
7	0			0			1		
8	1			0		0	0		
9	0	2·761	0·15	1	0·408		1	1·5	0·016
10	1			0			0		
11	0			0			0		
12	0			0			0		
Mean no. of larvae	1·386			0·733			1·133		
P_{χ^2}		>0·9			0·7–0·8			0·5–0·7	

It will be seen that the theoretical frequencies calculated according to Neyman agree fairly closely with the observed frequencies (P_{χ^2} 0·5–0·9).

IV. PERMANENT AND TEMPORARY PASTURES

As their names imply, permanent and temporary pastures differ in the length of their existence as grazings. The plant species on the two types are different; the species and strains of grasses and clovers of a temporary pasture are mainly those forms which are rich in protein but are not able to compete successfully with the ranker grasses which gradually supersede them if the pasture is not re-sown, and becomes 'permanent'. An old temporary pasture thus becomes almost indistinguishable from a permanent one.

Although comparison has been made of the microclimate formed by herbage of a few different plant species (Crofton, 1948*a*), detailed and extensive knowledge is lacking. Thus plant species differences cannot be used as a basis for comparing the suitability of permanent and temporary pastures as habitats of immature stages of trichostrongyles.

Associated with differences in plant species and age, however, is the amount of 'mat'. On temporary pastures there is little or no 'mat', and little or none is formed even at the end of a short ley. At the end of a long ley and on a well-established permanent pasture, the amount of 'mat' is considerable. The role of 'mat' in producing a microclimate favourable to the development and survival of trichostrongyles has been discussed previously (Crofton, 1948*a*, *b*), and it may be deduced that if other conditions are equal, permanent will harbour heavier infestations than temporary pastures.

Differences in age of pastures must necessarily have some effect in other ways. If we assume that conditions on a temporary pasture permit some overwintering of larvae and/or eggs, the frequency of ploughing will tend to limit population increases, even though this limitation is merely the result of reducing the amount of grazing by the hosts. The effect of ploughing as a physical factor will be discussed below.

Differences between permanent pastures and temporary pastures are thus obvious, but the evaluation of these differences in terms of fluctuations in populations of immature trichostrongyles is not simple, and further information is necessary. The observations described below are incomplete in many respects, but they were made in an attempt to study some of the problems.

(a) *Permanent pastures*

(i) *Pasture samples*, 1943–5

Samples were taken at approximately fortnightly intervals from three pastures during the months May to October in the years 1943–5. These fields will be referred to as A, B and C.

Field A was grazed mainly by store cattle but was also used for holding five or six horses for short periods; field B was grazed by sheep (ewes and their lambs) and cattle, while only ewes and lambs were pastured in field C. These three fields lay within 2 miles of one another, and it could be assumed that there was little variation between the fields with respect to climate. All three were on medium heavy soil and there were no gross differences in drainage. Apart from differences in types of stocking there was also considerable variation in management. While there were no signs of serious over-grazing, each field carried what was probably the maximum number of stock possible. When the farmers considered it necessary, they removed all the stock from a field for a week or 14 days or removed some of the animals for a similar period. No records of these changes in stocking are now available.

Figs. 1–3 show the results of sampling.

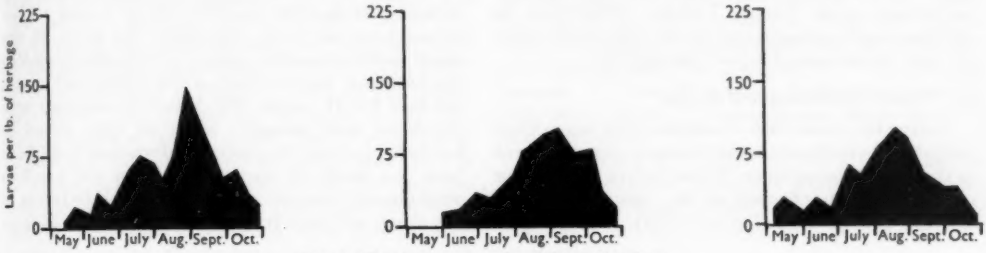

Fig. 1. Pasture counts for field A, 1943–5.

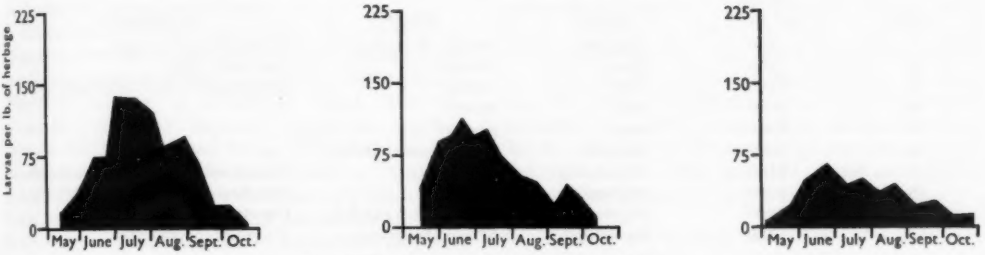

Fig. 2. Pasture counts for field B, 1943–5.

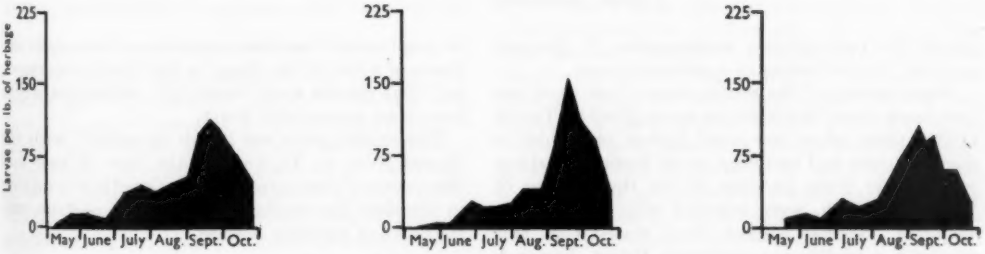

Fig. 3. Pasture counts for field C, 1943–5.

Two facts emerge from these results. First, the time of maximum infestation of the pasture differed not only from year to year but also from field to field; secondly, there is a greater variation in time of maximum infestation between fields in the same year than between years for the same field.

If only local climatic conditions controlled the time of maximum infestation, then less variation between fields might be expected. Variation between fields can be explained in many ways. First, the

microclimates are more important than macroclimates and no detailed comparison of microclimates was made. Secondly, the species of nematode larvae certainly differed from field to field, and even if these differences were merely numerical the work of Kauzal (1933), Stewart & Douglas (1938), Tetley (1935, 1941), Zvjaguintzev & Zawadowsky (1933) on seasonal incidence of different species indicates how differences in times of maximum infestation could occur. Thirdly, differences in stocking and management would effectively alter the rate of increase of larval populations.

(ii) *Pasture infestation and disease*

During the years 1940–8 samples were taken from pastures where clinical signs of disease were apparent in the stock grazing them. Table 2 gives the number of worms per lb. of grass on the pasture using the method described by Taylor (1939). Where determined by post-mortem examination of diseased animals, the predominant species are given.

Table 2

Farm	Larvae per lb.*	Month	Stock	Infestation
1	220	January	Sheep (Hoggets)	*Trichostrongylus* spp.
2	900	June	Sheep†	*Ostertagia* spp.
3	3300	July	Sheep†	*Haemonchus* and *Ostertagia* spp.
4	1200	July	Sheep†	?
5	1900	July	Sheep†	?
6	1800	August	Sheep† and cattle‡	?
7	3400	September	Sheep†	*Haemonchus* and *Ostertagia* spp.
8	3900	September	Sheep†	*Haemonchus* and *Ostertagia* spp.
9	2300	September	Sheep† and cattle‡	*Ostertagia* spp. and *T. axei*
10	300	December	Sheep (Hoggets)	*Trichostrongylus* spp.

* Mean of two samples taken at same time.
† Clinical symptoms in lambs only.
‡ Cattle unaffected.

Examination of the results shows that there are four cases which lie within the range given by Taylor (1939), three which are much higher, one which is slightly lower and two very much lower. The sheep grazing the three pastures where the number of larvae was high, were infected with *Haemonchus contortus*. Several workers have stated that this species is a prolific egg producer. Peters, Leiper & Clapham (1941), basing their estimates on those of Kauzal (1933), weighted the estimates of number of eggs per worm in mixed infestations to correct for differences in rate of egg production between species. The weighting figures they used were: *Nematodirus*, 1; *Ostertagia*, *Trichostrongylus*, *Cooperia*, Strongyloides, 10; *Oesophagostomum*, *Chabertia*, *Haemonchus*, *Bunostomum*, 100. The work of Kates (1947) suggests that the worm/egg ratios should be *Haemonchus* 1:1, *Trichostrongylus* 5·1:1, *Ostertagia* 1·8:1, *Cooperia* 5:1, *Nematodirus* 18·1:1, *Oesophagostomum* 1·2:1. Other workers have made less specific statements but are in general agreement that *Haemonchus contortus* has a higher rate of egg production than other trichostrongyles of sheep and cattle.

Thus the high counts in cases 3, 7 and 8 in Table 2 are understandable.

In cases 1 and 10, where the counts were much lower than Taylor's standard figures, further consideration is necessary.

Figures for farms 1 and 10 were obtained by sampling fields after an outbreak of winter trichostrongylosis had been reported. On farm 10 the sheep had been on the pasture for 5 weeks preceding the outbreak and on farm 1 the sheep had grazed the field for 11 weeks. The times of sampling were December and January, when no eggs would be hatching, so that the pasture infestation must have been the result of contamination in the previous autumn and bore no real relation to infestation of the sheep suffering from parasitic disease. It must be emphasized that the counts were made *after* the death of some of the sheep in the flocks concerned, and that counts made before the outbreaks might have been appreciably lower.

The results given are in fair agreement with the figures given by Taylor, but the time of year and the species of worm must be considered in attempting to correlate the number of larvae on a pasture with the onset of parasitic disease.

(*b*) *Temporary pastures*

In this section three types of pasture will be considered. First, the temporary pasture proper which is laid down as part of a system of crop rotation; secondly, newly made permanent pastures laid down after a period of arable cropping; thirdly, pasture produced by direct reseeding, that is, a pasture which is made as a result of sowing newly ploughed permanent pasture. Agriculturally these types differ considerably, particularly in relation to the general economy of the farm, but the differences to be considered here are mainly concerned with the

interval which elapses before the field is grazed again.

(i) *The effect of ploughing*

Obviously in the case of a pasture produced by direct reseeding the period of time which has elapsed since stock have grazed the field will be short—usually less than 9 months. Thus there is a possibility of a residual infection remaining to contaminate the new pasture. Several workers (Ackert (1923) and Payne (1922) on *Necator americanus*, Spindler (1936) on *Oesophagostomum dentatum*, Lucker (1936, 1938) on Horse Strongyles, Furman (1944) on *Ostertagia*) have shown that if infective larvae or faeces containing eggs are buried in soil there is subsequent migration of infective larvae to the surface. There is no reason to suppose that this is not also true for trichostrongyles, other than *Ostertagia*, and it is possible that direct reseeding does not produce a 'clean' pasture. The possibility that larvae could survive after several ploughings during a rotation is, however, a very remote one.

Direct evidence on these points was obtained by sampling new grass fields before stock grazed them. No larvae were detected on pastures which had been reseeded directly or otherwise. Each field was examined on three separate occasions, approximately 10 lb. of grass being examined on each occasion. It is not claimed, however, that the modified Taylor method will detect very low infestations, and the only conclusion which can be drawn is that these reseeded pastures were either free from infestation or that the infestation was so low as to be insignificant when compared with the chances of introducing infection by grazing so-called 'clean' stocks.

(ii) *Samples from reseeded pastures*

From the previous section it will be seen that examination of a reseeded pasture in the first year that it is grazed presents a unique opportunity to study the increase of larval populations, for the complication of residual infestations of either eggs or larvae which have overwintered is avoided. The initial infestations must influence the rate of increase of infestation, but where the initial infestation is low or absent, other factors will be more important. These factors are: the density and type of stocking; the degree of infestation of the introduced stock and the time of their introduction; the management of the pasture and, closely related to this, the ability of the pasture to provide a suitable environment for the development of the pre-parasitic stages.

The ability of the pasture to provide a suitable environment for development is determined by the physical state of the pasture, and particularly by the amount of 'mat' (Crofton, 1948*a*). As has been stated previously, young pastures have little or no 'mat'. In this respect a reseeded pasture is potentially less dangerous than an old one. Since, however, a young reseeded pasture is usually more productive than an old one the stocking of such a field is higher. Thus the rate of increase of larval populations may tend to be greater than on an old one because of increased stocking, whereas the lack of 'mat' will tend to reduce the longevity of the larvae and, indirectly, reduce the total population and the rate of increase. The position is still further complicated by the fact that the nutritional value of the herbage on a pasture is altered by reseeding, and this may alter the susceptibility of the grazing animals. The role of reseeding in parasite control is difficult to assess theoretically and direct observations are useful.

Observations were made on newly laid pastures to study the rate of increase of infective larvae. The fields chosen were representative of the different conditions and types to be found in the north of England.

Examination of Figs. 4 and 5 shows that in no case was the number of larvae per lb. of grass high. There appeared to be no strongly marked peak period of infestation, and the numbers fluctuated in an irregular manner. Admittedly, stocking changes were more frequent than on long-established pastures, but, as will be seen from the next section, this is only part of the reason for the fluctuations. The figures give some measure of the availability of the infective larvae to the grazing animals.

(iii) *Effect of grass length on estimates of populations*

A grass seeds mixture contains the seeds of a large number of plants. One of the reasons for the use of a mixture is that different species have different times of maximum productivity, and the mixture is so balanced that there is a succession of dominance of different species in an attempt to maintain even growth throughout the longest possible period. The object of a seeds mixture would not, however, be attained if only periods of maximum growth were considered, and an attempt is made to maintain a satisfactory protein-carbohydrate balance during the main grazing season. This attempt to achieve a nutritional balance interferes to some extent with the gross productivity of the pasture so that there is not, even under ideal conditions, a uniform growth throughout the year. Added to this, variations in climatic conditions cause successive periods of growth and non-growth of different plants, so that the amount of herbage available for grazing animals changes from time to time. The farmer is familiar with 'flushes' and 'checks' and attempts, so far as is possible, to regulate the grazing of his fields.

This variation in growth of herbage must affect the availability of larvae to the grazing animal, and Taylor (1935) has discussed this in relation to the etiology of winter trichostrongylosis. The results

presented in the previous section give a measure of the availability of infective larvae to grazing animals, the number of larvae per lb. of herbage being plotted. These figures do not necessarily give a measure of the total number of infective larvae on the pasture at different periods, and the graphs do not give a measure of actual production of larvae. Using the technique described in a previous paper (Crofton, 1949) a correction figure for the length of grass was calculated for each time of sampling (see previous section). The results given in Figs. 4 and 5 were weighted by applying the appropriate correction figure and the new curves plotted (a discussion of the validity of this is made in Crofton, 1949).

As can be seen, this produced a much smoother set of curves, although there was still the considerable variation which might be expected as a result of changes in stocking. The corrected figures show an irregular but definite increase with increased age of pasture, until September when the sampling was stopped. These figures represent the total population of the pasture, giving a more accurate measure of the pasture contamination than the uncorrected figures.

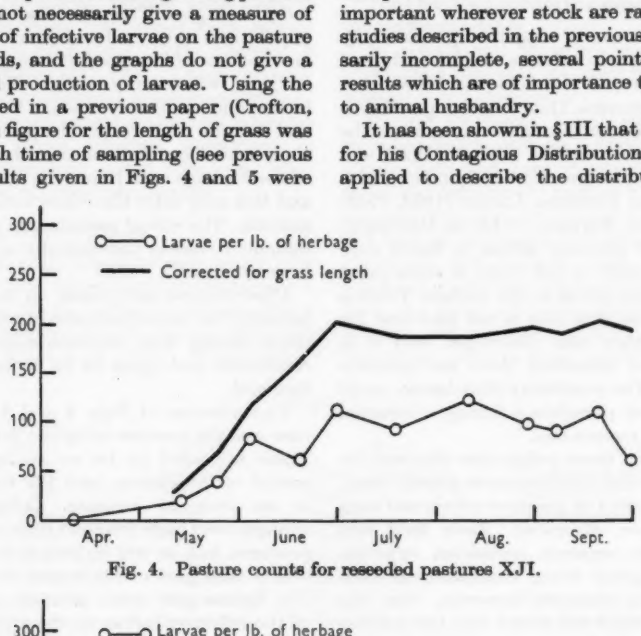

Fig. 4. Pasture counts for reseeded pastures XJI.

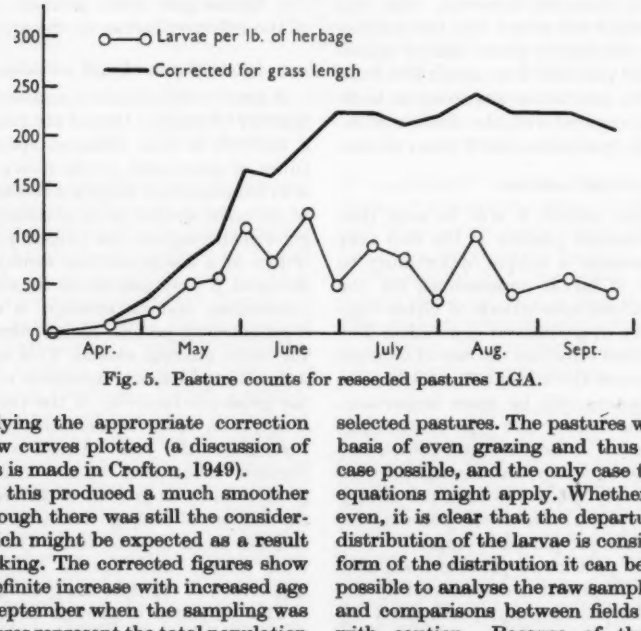

Fig. 5. Pasture counts for reseeded pastures LGA.

V. DISCUSSION

The problems of infestation of lowland pastures are important wherever stock are raised, and while the studies described in the previous sections are necessarily incomplete, several points emerge from the results which are of importance to further work and to animal husbandry.

It has been shown in § III that Neyman's equation for his Contagious Distribution Type A could be applied to describe the distribution of larvae on selected pastures. The pastures were selected on the basis of even grazing and thus gave the simplest case possible, and the only case to which Neyman's equations might apply. Whether or not grazing is even, it is clear that the departure from a random distribution of the larvae is considerable. From the form of the distribution it can be seen that it is not possible to analyse the raw sampling data efficiently and comparisons between fields must be accepted with caution. Because of these difficulties no statistical analysis of pasture counts has been attempted here, and only broad conclusions have

been drawn. The analysis of pasture counts will be considered in another paper. It will be noted that, with the exception of figures given in §IV (*a*) (ii), conclusions are related only to *periods* of maximum infestation and to general trends.

In §IV (*a*) (ii) there is some justification for considering more detailed counts, although no strict interpretation has been made. Taylor (1939) suggested a criterion by which the dangers of parasitic disease could be assessed by pasture samples. There are more certain and accurate ways of diagnosing subclinical parasitosis, and Taylor did not intend that his method should be used for precise diagnosis; his technique for recovery of larvae from pastures has its interest and uses however.

Without attempting to draw precise conclusions from the individual data obtained by sampling permanent pastures, two points are immediately apparent: (1) the time of maximum infestation of the pastures differed from year to year; (2) the variation in the time of maximum infestation between fields is greater than the variation between years. Taking into consideration the fact that the fields sampled were close to one another, these points are of considerable interest. Despite differences in microclimate the variation in time of maximum infestation between fields cannot be completely explained in terms of pure ecology. Differences in management and stocking must play some part, and this has been widely recognized because attention to management and stocking has been one of the major principles involved in parasite control. That management and stocking could affect the time factor so considerably may not have been so clearly evident. In cases of epidemics the variation in times of different outbreaks has been accepted. If ecology were the only factor involved one might expect all outbreaks in a small area to occur at the same time, but this does not always happen. While climatic conditions determine the relative incidence of different species of parasite, and we recognize that different species predominate in outbreaks at different periods of the year, the fact that not all farms in the same area suffer these outbreaks must indicate that climatic conditions are not the only factors involved. Studies in pure ecology are therefore not sufficient, and work along these lines must be combined with a study of population. Gordon (1948) suggests that '...it might be more desirable to think of epidemiology in terms of population dynamics'.

The work on temporary pastures shows that in the cases considered ploughing effectively produced clean pastures. Whether or not any infective stages remained after ploughing cannot be decided, but if there were larvae remaining on the pastures the numbers were too small to be detected.

On reseeded pastures the concentration of larvae on the herbage remained low despite the increased rate of stocking which usually occurs after a field has been successfully reseeded. Making allowances for the length of the grass at different times of sampling it was shown that the total population of larvae was considerable, and that the number of larvae which could potentially overwinter was high. Examination of reseeded pastures in their second year would be profitable. With a high total population of larvae at the end of the first grazing season, it seems possible that in the second year the number of larvae will be dangerously high in a field which must be heavily grazed.

The problem of studying concentrations of larvae rather than numbers is not a simple one, and some of the difficulties are mentioned in a previous paper (Crofton, 1949). It is, however, an important study which must be included in any consideration of population on a pasture. Taylor (1935) has discussed *grass length* in relation to disease outbreaks, but the case he quotes, that of a pasture after a long period of drought, is an extreme one, and further investigation of more normal pastures at different seasons might be profitable.

VI. SUMMARY

1. Permanent and temporary pastures were examined using a modification of Taylor's method.

2. The goodness of fit of Neyman's Contagious Distribution Type A when applied to larvae on pastures is discussed.

3. Examination of permanent pastures over a period of 3 years showed that the difference in time of maximum infestation was greater between fields in the same year than between years for the same field.

4. Counts made on pastures where disease had occurred showed reasonably close agreement with Taylor's estimate.

5. No larvae were detected on fields after ploughing and reseeding.

6. The number of larvae per lb. of herbage on reseeded pastures was low, but the growth of grass masked the fact that the total population was relatively high at the end of the season.

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ON THE SYSTEMATIC POSITION OF THE GENUS *DEROPRISTIS*, OF *DIHEMISTEPHANUS STURIONIS* LITTLE, 1930, AND OF A NEW DIGENETIC TREMATODE FROM A STURGEON

By R. M. CABLE, *Department of Biological Sciences, Purdue University, Lafayette, Indiana, U.S.A.*

(With 8 Figures in the Text)

In April 1950, two specimens of the shovel-nosed sturgeon, *Scaphirhynchus platorhynchus* (Rafinesque), were taken from the Wabash River near Lafayette and examined for helminth parasites. Both were infected with trematodes representing a new genus and species closely related to *Deropristis*, species of which occur in eels, sturgeons and possibly other migratory fishes. Although considered to belong to the family Acanthocolpidae by other investigators, the genus *Deropristis* was removed from that family by Cable & Hunninen (1942) in their study on the morphology and life cycle of *D. inflata*. By excluding also *Dihemistephanus sturionis*, it was possible to redefine the Acanthocolpidae rather precisely. In that paper, doubt was expressed that *D. sturionis* belonged to either the genus *Dihemistephanus* or the family Acanthocolpidae, but because of certain features, no disposition of the species was made. A new subfamily, Deropristiinae, was proposed and placed in the Lepocreadiidae to contain *Deropristis* whose life history and morphology suggested closer affinities with the lepocreadiids than with the Acanthocolpidae. Dawes (1946, 1947) has rejected this proposal, however, retaining the classification based on the views of earlier investigators.

The writer's observations on the trematode described below indicate that this species, *Dihemistephanus sturionis* and species of *Deropristis* are so closely related that, should their differences indeed be of generic value, these forms should be united in the nearest suprageneric category, i.e. subfamily, unless one employs Poche's (1925) complicated system. This may be accomplished more simply, however, by emending the Deropristiinae to include these trematodes, although the family position of that subfamily is debatable, as will become apparent in later discussion.

METHODS

The two fish were obtained alive, killed and examined immediately. The worms were removed to isotonic saline and held there for several hours to supply living specimens for observations on the excretory and reproductive systems. Details of the female complex were determined readily in this material when viewed from the dorsal side. The remaining worms were killed under light pressure in alcohol-formol-acetic fixative, stained with Semichon's carmine and prepared as whole mounts. Unless otherwise stated, figures were drawn by microprojection except for the finest details which were added free-hand in somewhat stylized form.

OBSERVATIONS AND DISCUSSION

Much repetition may be avoided by first emending the definition of the subfamily as follows:

Subfamily Deropristiinae
Cable & Hunninen, 1942, *emend.*

Diagnosis. Elongate distomes with moderately developed ventral sucker within the anterior one-fourth of the body. Cuticle with extremely prominent spines arranged quincunxially in transverse rows and becoming progressively farther apart toward the posterior end. Remnants of the cercarial eye-spots present. Mouth ventral, subterminal; oral sucker well developed; prepharynx short, pharynx prominent; oesophagus short, receiving the caeca at a level well anterior to the ventral sucker. Caeca tubular, simple, and extending almost to the posterior end of body. Genital pore midventral, just anterior to the ventral sucker. Genital atrium unarmed, tubular, and either very short or extending well posterior to the ventral sucker. Cirrus-sac well developed; cirrus and prominent metraterm armed with conspicuous spines which, although slender and sharp pointed, appear flexible and easily deflected. Pars prostatica weakly developed; prostatic cells inconspicuous; seminal vesicle bipartite with an intervening septum. Testes two in number, oval or slightly pyriform, smooth, and located in the posterior body region, in tandem or slightly diagonal, overlapping or well separated. Vitelline follicles rather scanty, beginning at the level of the seminal vesicle and not extending into the extreme posterior end of the body. Ovary smooth, located near or posterior to the middle of the body. A true seminal receptacle adjacent to the ovary. Laurer's canal

present, opening dorsally to the left of the mid-line. Uterus extends from the ovary first posteriorly as far as, or beyond, the testes and then anteriorly to join the metraterm. Eggs numerous, medium in size, and operculate. Excretory vesicle short, tubular or sac-like, with a pair of main excretory tubules extending from its anterolateral margins, each receiving an anterior and a posterior collecting tubule at the level of the ventral sucker or (?) extending into the cervical region before being joined by secondary tubules; six groups of flame cells on each side. Adults parasitic in the intestine of migratory fishes, principally eels and sturgeons. The cercaria, so far as is known, is biocellate, with an ornate tail and without a stylet; it develops in simple rediae in prosobranch gastropods and encysts in invertebrates.

TYPE GENUS. *Deropristis* Odhner, 1902.

Includes also: *Pristotrema* n.g., *Pristicola* n.g. (syn. *Dihemistephanus* Little, 1930, *nec* Looss, 1901).

The type genus, *Deropristis*, is well known from the accounts given by Odhner (1902), Manter (1926), Ward (1938) and Cable & Hunninen (1942). However, it is characterized briefly, and a figure of *D. inflata* is included in this paper for comparison with the other genera assigned to the subfamily.

Deropristis Odhner, 1902 (Fig. 1)

DIAGNOSIS. With the characters of the subfamily Deropristiinae. Forebody concave ventrally and with prominent lateral expansions bearing conspicuously enlarged spines; a mid-dorsal patch of similar spines at about the pharyngeal level. Genital atrium long and tubular, extending well posterior to the ventral sucker. Ovary near or posterior to the mid-level of the body. Uterus extends into the space between the ovary and the anterior testis, which it may partly overlap. Vitelline follicles distributed from the level of the seminal vesicle to a point between the ovary and the anterior testis. Testes close together or overlapping. Includes: *D. hispida* (Abildgaard), type; *D. inflata* (Molin).

The new trematode now to be described differs from *Deropristis* in a number of respects, some of which are of no more than specific value. However, taken collectively, they are considered to be of generic significance.

Pristotrema n.g.

DIAGNOSIS. With the characters of the subfamily Deropristiinae. Body narrower anterior to ventral sucker than posterior to it, tapering slightly toward the oral sucker. Cuticular spines nowhere abruptly enlarged in patches or rows. Genital atrium short, not extending to the posterior edge of the ventral sucker. Vasa efferentia unite just before entering the cirrus-sac; testes well separated. Ovary near the mid-level of body. Uterus extensively coiled, filling the space between the testes and overlapping the posterior testis or even extending into the extreme posterior end of body. Vitelline follicles may reach the posterior edge of the anterior testis.

Type and only species:

Pristotrema manteri n.sp. (Figs. 3–8)

DIAGNOSIS. With the characters of the genus. Spines largest on the forebody, becoming progressively much smaller in succeeding rows anterior to the pharynx and decreasing gradually in size and number toward the posterior end of body. Measurements taken from whole mounts of specimens varying from those with only a few eggs in the uterus to the most mature individuals show the following ranges in size: body length 3·44–12·38 mm., maximum width 0·6–1·95 mm. Ventral sucker spherical or, in contracted specimens, slightly oval, 0·165–0·393 mm. in diameter. Prepharynx shorter than the pharynx, up to 0·135 mm. long; pharynx 0·158–0·304 mm. long and 0·104–0·255 mm. wide. Oesophagus about the length of the pharynx in moderately extended specimens. Genital atrium extremely short, not extending posteriorly dorsal to the ventral sucker. Cirrus-sac 0·470–1·14 mm. long and 0·174–0·450 mm. in maximum width at the level of the seminal vesicle. Anterior division of the seminal vesicle usually larger than the posterior portion and possibly concerned with spermatophore formation. Ovary oval or pyriform, measuring from 0·276 by 0·242 to 0·662 by 524 mm. Oviduct leaves the ovary on the right side of its dorsal surface, extending to the left margin of the ovary, where it receives a common opening from Laurer's canal and the seminal receptacle, situated just posterior to the ovary. Laurer's canal opens dorsally and to the left as far as the caecum of that side. Oviduct then turns anteriorly, receiving the vitelline duct at about the anterior margin of the ovary. Ootype dorsal and just anterior to the ovary. Mehlis's gland inconspicuous and poorly developed. From the ootype the uterus bends abruptly and extends posteriorly, filling the space between the testes and overlapping the posterior testis, or even extending into the extreme posterior body region; the uterus then turns and extends anteriorly in a tortuous path to join the metraterm, which is about equal to the cirrus in length, measuring 0·186–0·590 mm. long. Testes oval, reniform, elliptical or pyriform in outline, depending on the state of contraction; anterior testis from 0·47 by 0·317 to 1·52 by 0·894 mm.; posterior testis from 0·586 by 0·3 to 2·14 by 1·2 mm. Eggs 0·043 to 0·052 mm. long and 0·022 to 0·026 mm. wide. Excretory vesicle barely reaching the posterior testis and with conspicuous radiating muscle fibres; flame cells numerous, but the pattern undetermined.

DEFINITIVE HOST. The shovel-nosed sturgeon, *Scaphirhynchus platorhynchus* (Rafinesque) and possibly the lake sturgeon, *Acipenser rubicundus* Le Seur.

SITE. Spiral valve of the intestine.

TYPE LOCALITY. Wabash River, Indiana, U.S.A.

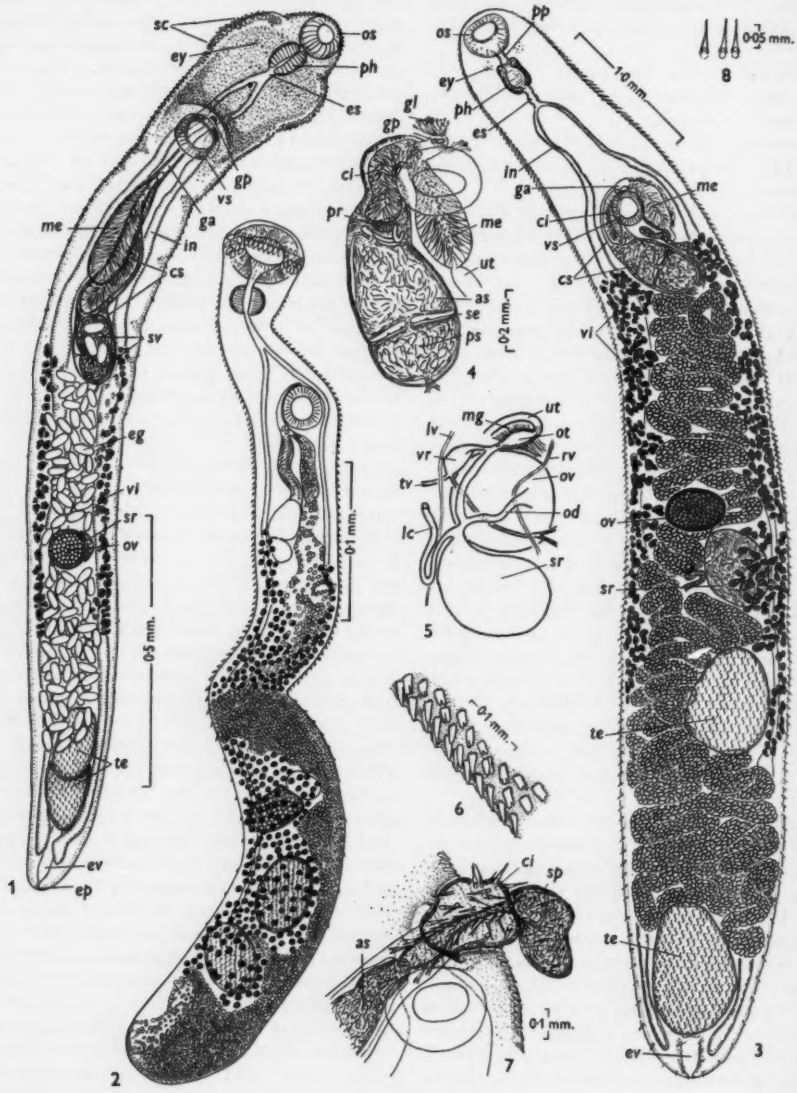
LIFE HISTORY. Unknown.

TYPE SPECIMENS. Holotype no. 37186 and paratypes no. 37187, Helminthological Collection, U.S. National Museum.

That *Pristotrema manteri* may occur in the lake sturgeon is suggested by Ward's (1938) reference to trematodes which Stafford (1904) reported as '*Deropristis hispidus*' from that fish. As Ward stated, the sucker ratio, extent of the uterus, and failure to mention the prominent cervical expansions, indicate that Stafford was dealing with a form that does not even belong to the genus *Deropristis*. On the other hand, Stafford's brief description is in agreement with the diagnosis of the genus *Pristotrema* given above, and it seems very likely that he may have had *P. manteri*. Ward mentioned a specimen in his collection which was taken from the lake sturgeon and was very similar if not identical with the species Stafford had. Ward expressed the intention of returning to this form in a later paper, but apparently did not accomplish this before his death in 1945. Dr E. W. Price has kindly searched through the Ward Collection in an unsuccessful attempt to locate this material. It may be found when the Collection is catalogued, a task that is about half completed. In a re-study of Stafford's Collection, Miller (1941) gives no additional information about the species found in the lake sturgeon and considered in this paper as very possibly being *P. manteri*.

One of the fish examined by the writer contained fifteen specimens of *P. manteri*, all rather uniformly intermediate in size, while the other harboured a much larger number which varied from less than 1·0 to over 12·0 mm. in length. The smallest were immature and were evidently recently excysted metacercariae, because concretions precisely like those in the cercaria of *Deropristis inflata*, closely related to *Pristotrema manteri*, were still present in the excretory vesicle. Sexual maturity, the criterion of which is the presence of eggs in the uterus, is attained long before the species reaches its maximum size. It should be noted that the posterior extent of the uterus is not dependent on the number of eggs therein. The smallest mature specimen contained only a few dozen eggs and these were in uterine coils alongside the anterior portion of the posterior testis, whereas the pre-ovarian loops of the uterus were empty. Furthermore, in two specimens considerably smaller than the largest obtained, the uterus extends into the extreme posterior end of the body.

The possibility of spermatophore formation in *P. manteri* was mentioned in the specific diagnosis and should be discussed further. In a number of worms placed in saline for a few hours it was observed that what appeared with the unaided eye to be the cirrus was conspicuously everted. These specimens were fixed, some under sufficient pressure to roll and flatten them until a lateral view of the protruded cirrus would be obtained in the whole mount. When the specimens were stained and examined, it was found that a large part of what had been judged to be the cirrus was actually a well-defined mass of sperms (Fig. 7), continuous with those in the anterior portion of the seminal vesicle. The protruding mass appeared to be enclosed in an extremely thin membrane, and, in one specimen in which the mass had ruptured, remains of this membrane were distinctly seen. Because the worms had been very active in the saline, it is unlikely that sperms emitted from the cirrus would remain in such a compact, well-defined mass unless they were restrained. Thus it appears certain that the membrane is not a coagulation artifact resulting from fixation. Odhner (1905, 1911), and more recently Crowcroft (1947, 1950), have described the formation of stalked spermatophores in trematodes belonging to the families Prosorhynchidae and Fellodistomidae. It is interesting to note that these, like genera of the Deropristiinae, possess a bipartite seminal vesicle and glands opening near the genital pore. Odhner and Crowcroft at first attributed the formation of the spermatophore to the glands of the genital atrium, but later expressed the view that the pars prostatica perhaps played the major part. This does not seem at all likely in the present species, because of its poorly developed prostatic complex. The situation here suggests yet another source of the spermatophore, viz. the anterior division of the seminal vesicle. In all the writer's specimens, the wall of the cirrus-sac enclosing this portion of the vesicle contains very small, intensely staining nuclei in finely granular cytoplasm of glandular appearance. This condition does not extend posterior to the septum dividing the seminal vesicle. Some specimens show continuity of the sperms in the two portions of the vesicle (Fig. 4), whereas others do not. Furthermore, this connexion, when it is present, consists apparently of sperms alone, indicating that the wall of the vesicle is closely applied to the septum at that point. Crowcroft (1950) has reported the presence of a narrow sphincter between the two portions of the seminal vesicle of *Lintonium consors*. Evidence of such a sphincter has been observed in living specimens of *Pristotrema manteri*, in which a distinct constriction of the cirrus-sac at the level of the septum was noted. From these observations it seems that the spermatophore may be formed in the following manner. The anterior portion of the vesicle fills with sperms passing through the orifice of the septum from the posterior chamber. Constriction of the



Figs. 1–8.

Fig. 1. *Deropristis inflata* (Molin), ventral view. From Cable & Hunninen (1942).

Fig. 2. *Pristicola sturionis* (Little) n.g., n.comb., ventral view. Redrawn from Little (1930).

Fig. 3. *Pristotrema manteri* n.g., n.sp., ventral view of holotype.

Fig. 4. *P. manteri*, terminal genitalia.

Fig. 5. *P. manteri*, female complex drawn free-hand from dorsal view of living specimen.

Fig. 6. *P. manteri*, detail of spination in cervical region.

Fig. 7. *P. manteri*, specimen with everted cirrus and spermatophore.

Fig. 8. Spines of cirrus.

cirrus-sac may then close the orifice and the spermatophore forms in the anterior chamber, possibly under the influence of a secretion from the wall of the cirrus-sac anterior to the septum.

In view of the morphology of *P. manteri* and especially its variability in the extent of the uterus, it is now possible to give critical consideration to *Dihemistephanus sturionis* Little, 1930, a species whose systematic position has been much in doubt. Cable & Hunninen (1942) excluded this trematode from the Acanthocolpidae, but made no further disposal of it, because of the extent of the uterus and vitelline follicles, as well as certain features of the excretory system as described by Little. That this species is very closely related to *Deropristis* and *Pristotrema* seems certain from similarities in both the morphology and the host-parasite relationship. In the extent of the vitellaria and uterus, *Pristotrema manteri* is intermediate between *Deropristis* and *Dihemistephanus sturionis*. Little described the excretory system as being Y-shaped with a short basal portion and long arms extending to the level of the pharynx, thus agreeing with the arrangement described for certain acanthocolpids and apparently differing from what has been observed in *Deropristis* and *Pristotrema*. Little's observations were based on serial sections of two specimens, instead of on the much more favourable living material, and his figures of cross-sections show the arms of the system to be of a size that indicates that they are main excretory tubules, while the short basal stem is the vesicle. Because in both *Deropristis* and *Pristotrema*, the main tubules extend only to the ventral sucker, it may be wondered whether Little may have overlooked in sections a division of the main tubule in that vicinity to form anterior and posterior collecting tubules. In many of the writer's whole mounts of *P. manteri*, the anterior collecting tubule in the forebody is much more distinct than is any other part of the excretory system except the vesicle. In the cercaria of *Deropristis inflata*, the writer was deceived by the manner in which the tubule of the third flame cell group turned at the pharyngeal level and extended posteriorly, giving the appearance that it was the main tubule. However, should the excretory system of *Dihemistephanus sturionis* be as described, it is not by itself sufficient basis for regarding the species as being co-familial with such acanthocolpid genera as *Stephanostomum* and *Acanthopsolus* in which, unlike *Dihemistephanus sturionis*, the prepharynx is very long, the seminal vesicle is not bipartite, sperms are stored in a receptaculum uterinum or in the uterus instead of in a true seminal receptacle, vitelline follicles extend to the posterior end of the body and the uterus is entirely pre-ovarian. Furthermore, spination of the cirrus and metraterm, when it is present in acanthocolpids, is of a different type from that which is so uniformly alike in *Deropristis*, *Pristotrema* and *Dihemistephanus sturionis*. Also, the fact that these three all occur in sturgeons emphasizes their close relationship and points to a parallel evolution of parasite and host. In a personal communication, Caballero agrees essentially with this view in stating, in a forthcoming paper, his intention of excluding *Deropristis* and *Dihemistephanus sturionis* from the Acanthocolpidae. He is also excluding *D. sturionis* and *D. brachyderus* from the genus *Dihemistephanus*, proposing a new genus for *D. brachyderus*, but retaining it and *Dihemistephanus* in the Acanthocolpidae.

Although *Dihemistephanus lydiae*, type of the genus *Dihemistephanus*, has never been adequately characterized, the descriptions given by Looss (1901) and Nicoll (1909) indicate that *D. sturionis* is not to be identified with the genus *Dihemistephanus* which, as Looss stated, is very similar to *Stephanostomum* (syn. *Stephanochasmus*), and is here

Key to lettering on figures

as	anterior chamber of seminal vesicle	*ot*	ootype
ci	cirrus	*ov*	ovary
cs	cirrus sac	*ph*	pharynx
eg	egg	*pp*	prepharynx
ep	excretory pore	*pr*	prostate
es	oesophagus	*ps*	posterior chamber of seminal vesicle
ev	excretory vesicle	*rv*	right vas efferens
ey	remnants of eye-spots	*sc*	cervical spines
ga	genital atrium	*se*	septum
gl	glands of genital atrium	*sp*	spermatophore
gp	genital pore	*sr*	seminal receptacle
in	intestine	*sv*	seminal vesicle
lc	Laurer's canal	*te*	testis
lv	left vas efferens	*tv*	transverse vitelline duct
me	metraterm	*ut*	uterus
mg	Mehlis's gland	*vi*	vitellaria
od	oviduct	*vr*	vitelline reservoir
os	oral sucker	*vs*	ventral sucker

regarded as belonging to the Acanthocolpidae. According to Little (1930) *Dihemistephanus sturionis* lacks eye-spot pigment. In *Pristotrema manteri* this pigment is very diffuse and, being by no means as noticeable as in the true acanthocolpids, would have been overlooked, had a special search for it not been made. In re-defining the genus *Dihemistephanus*, Little relied more on the characters of *D. sturionis* than on descriptions of the type species, *D. lydiae*, on which the genus is based. A point by point comparison of descriptions of *D. lydiae* and *D. sturionis* convinces the writer that the two are not congeneric, *D. sturionis* being on the whole much more like *Deropristis* and *Pristotrema* than *Dihemistephanus lydiae*. For that reason it is proposed that a new genus be erected for *Dihemistephanus sturionis* and that this genus be allocated to the subfamily Deropristiinae.

Pristicola n.g. (Fig. 2)

Syn. *Dihemistephanus* Little, 1930, *nec* Looss, 1901.

Diagnosis. With the characters of the subfamily Deropristiinae. Body wider in the cephalic than in the cervical region. Oral sucker much larger than the ventral sucker. Spines on the forebody becoming progressively smaller anterior to the pharynx, except for a very prominent, double row of flat, almond-shaped spines encircling the anterior end at the mid-level of the oral sucker, except for a wide ventral gap. Genital atrium moderately long and tubular, reaching the posterior edge of the ventral sucker. Ovary well posterior to the mid-level of the body. Vitellaria extend to a point alongside or slightly beyond the posterior testis. Uterus voluminous, reaching the extreme posterior end of the body. Testes slightly separated.

Type and only species. *Pristicola sturionis* (Little, 1930) nov. comb., syn. *Dihemistephanus sturionis* Little, 1930.

Ward (1938) commented on trematodes described by Van Beneden (1871) as 'young stages' of *Deropristis hispida*. Since these were figured as possessing 'a characteristic echinostome cephalic crown of spines', Ward was of the opinion that they were some sturgeon parasite other than *Deropristis*. In view of their structure it seems very likely that they may have been the species described later by Little and here assigned to the genus *Pristicola*.

Of the three genera placed in the Deropristiinae, *Pristotrema* is the most generalized so far as spination is concerned. From this type the other two are easily derived. By lateral expansion of the cervical region and the enlargement of the quincunxially arranged spines there and on the mid-dorsal region of the forebody, spination as in *Deropristis* results. Indeed, this sequence of events occurs during post-cercarial development of *D. inflata* as described by Cable & Hunninen (1942). On the other hand, enlargement of two rows of cephalic spines, together with decrease in the size of others in that region, would result in the condition seen in *Pristicola*. Furthermore, the existence of such diverse spination in three genera, which otherwise are so similar, emphasizes the caution that should be employed in evaluating cephalic and circumoral spination as indications of relationship. There seems, for example, insufficient basis for postulating, as has been done, echinostome affinities of the Acanthocolpidae largely on the basis of cephalic spines.

Although the genera *Deropristis*, *Pristotrema* and *Pristicola* form a natural group of closely related forms here placed in a separate subfamily, it is not clear as to what family the group should be assigned. When the Deropristiinae was proposed, Cable & Hunninen (1942) allocated it to the Lepocreadiidae, which they regarded as being distinct from the Allocreadiidae. They restricted the Allocreadiidae to trematodes which have ophthalmoxiphidiocercariae developing in bivalve molluscs, because Looss (1894) had described such a larva for *Allocreadium isoporum*, type species of the type genus. Recently, however, Seitner (1951) has traced in this laboratory the life cycle of another species, *A. ictaluri*, whose development he found to be remarkably similar to that of *Deropristis* and the 'Anallocreadiine' larvae described by Hopkins (1937). The cercaria of *Allocreadium ictaluri* lacks a stylet and develops in a prosobranch gastropod, whereas the larva supposed by Looss (1894) and Dollfus (1949) to be that of *A. isoporum* has a stylet and develops in sphaeriids. Seitner found it difficult to believe that two species in the same genus could differ to this extent, and, because neither Looss nor Dollfus demonstrated the life cycle of *A. isoporum* experimentally, he chose to regard that cycle as unproved. With this view the writer agrees; on the basis of morphological comparisons, the ecology of the larva and the adult, and present knowledge of the life cycle of *Deropristis inflata*, it would appear that the cercaria Seitner had was more likely to be the larva of *Pristotrema* than of a species of *Allocreadium*. Seitner's study, then, indicates a closer relationship between the Allocreadiidae and Lepocreadiidae than was expected, and his comparison of the miracidia of *A. ictaluri* and *Crepidostomum ictaluri*, in addition to differences in their cercariae and molluscan hosts, suggests that the papillose forms and *Allocreadium* may be so distantly related as to require their eventual allocation to distinct families. The structure of the excretory vesicle in the ophthalmoxiphidiocercariae is not at all like that of the larvae of *Allocreadium ictaluri* and *Deropristis inflata*, being more like that of gorgoderid cercariae. The problem of relationship between the Lepocreadiidae and *Allocreadium* cannot be resolved, however, without further know-

ledge of life histories, the most important of which is the experimental determination of the cycle of *A. isoporum*. Ultimately, it may become necessary to return the Lepocreadiidae to the Allocreadiidae, with or without retaining *Crepidostomum* and related genera in the combined group. The difficulty at present is a question of the interrelationships of all the trematodes at one time or another assigned to the Allocreadiidae *sensu lato*, and many of the families more recently proposed for species occurring in fishes. Although the problem is more complex, it may be compared to that which existed 20 years ago in respect to the Heterophyidae and Opisthorchiidae, now generally recognized as belonging to a common superfamily, if indeed they should not be considered as subfamilies.

Should the precedent set by other investigators in erecting families for small groups on the basis of adult structure be followed, the Dercpristiinae would be elevated to family rank. There is no existing family to which its members may be allocated finally. In several respects, including the bipartite seminal vesicle and scanty vitellaria, they resemble the Steringophoridae, but in what may be more important respects, especially the excretory system and life cycle, they do not. Their chief resemblance to the Acanthocolpidae is the spiny body and genitalia. Although they differ from the lepocreadiids in the same respects in which they resemble Steringophorids and acanthocolpids, there is at present as much basis for allocating the Dercpristiinae to the Lepocreadiidae as to any other family. Because this disposition of the group has been made by Cable & Hunninen (1942), it is retained until further information is at hand.

SUMMARY

Pristotrema manteri n.g., n.sp. is described from the spiral valve of the shovel-nosed sturgeon, *Scaphirhynchus platorhynchus* (Rafinesque), in the Wabash River, Indiana, U.S.A.

A new genus, *Pristicola*, is erected for *Dihemistephanus* Little, 1930, *nec* Looss, 1901, with *Pristicola sturionis* (Little) as the type and only species.

The subfamily Dercpristiinae Cable & Hunninen, 1942, is re-defined to receive the genera *Pristotrema* and *Pristicola* as well as the type genus, *Dercpristis*.

The uncertain family position of the Dercpristiinae is discussed without removing it from the Lepocreadiidae, although the independent status of that family may be questioned in the light of recent work.

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THE STRUCTURE OF THE ADULT AND LARVAL STAGES OF *PLAGIORCHIS* (*MULTIGLANDULARIS*) *MEGALORCHIS* N.NOM. FROM THE TURKEY AND AN EXPERIMENTAL DEMONSTRATION OF THE LIFE HISTORY

By GWENDOLEN REES, D.Sc., *Department of Zoology, University College of Wales, Aberystwyth*

(With 46 Figures in the Text)

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I. INTRODUCTION

On 8 June 1950 four turkey poults, 2 weeks old, from a total of 13 which had died on a farm in Radnorshire, were sent to Mr L. E. Hughes and Mr F. Jordan at the Veterinary Investigation Laboratories, Aberystwyth. Post-mortem examinations revealed the presence of very large numbers of trematodes in the duodenum and small intestine of each bird. The trematodes were handed to the writer for identification, and were found to belong to the genus *Plagiorchis* Lühe, 1899. A similar condition has been reported once previously, by Foggie (1937) in Northern Ireland, where a fatal outbreak of parasitic necrosis was found in turkey poults. Foggie identified the trematode provisionally as *P. laricola* Skrjabin, 1924. The specimens found in the present investigation are identical with those found by Foggie. The writer is indebted to Dr D. O. Morgan of the University of Edinburgh for the loan of some of Foggie's slides and material for comparison. Unfortunately, neither Foggie nor the writer have been able to obtain the original description of *P. laricola*.

The genus *Plagiorchis* Lühe, 1899, has been much studied. Massino (1929) gave a detailed account of the genus, described some new species and included a key for the identification of 25 of the species then known. A translation from the Russian of this key was very kindly lent by Foggie. Using the key, the present species and that of Foggie can be identified as *P. laricola*, the diagnostic features given being the ovary is larger than the ventral sucker, the vitellaria are in front of, or on a level with, the forking of the gut and join in the middle line anteriorly, and the cirrus-sac does not extend beyond the posterior margin of the ovary. If, however, all the known species of *Plagiorchis* are considered these characters are common to some other species as will be shown later. Schulz & Skworzow (1931) described in detail *P. arvicolae* from the water rat and divided the genus into two subgenera, namely, *Plagiorchis*, in which the vitelline follicles in the region anterior to the acetabulum are confined to the lateral regions of the body with at most only a few follicles extending to the median line, and *Multiglandularis*, in which the vitelline follicles in the region anterior to the acetabulum extend across the body as a broad continuous band or commissure.

The present species belongs to the subgenus *Multiglandularis*. Schulz & Skworzow included 24 species in the subgenus *Plagiorchis* and 19 in the subgenus *Multiglandularis*. They listed a further 7 species, about which insufficient was known to place them with certainty in either subgenus. This made a total of 50 known species by 1931. Olsen (1937), in a systematic study of the subfamily Plagiorchiinae Pratt, 1902, included a key to the two subgenera and species of *Plagiorchis*, placing 25 species in the subgenus *Plagiorchis* and 20 in the subgenus *Multiglandularis*. In addition 4 further species were mentioned but not included in the key as no descriptions of them were available. Olsen's paper therefore included 49 species. He omitted 6 species mentioned by Schulz & Skworzow, so that the total of known species in 1937 was 55. Using Olsen's key the species now being considered does not run down to *Plagiorchis* (*M.*) *laricola* nor to any of the others. Since 1937, a further 12 species of *Plagiorchis* have been described making a total of 67 species to date. Of these, 29 fall into the subgenus *Multiglandularis*; descriptions of 25 are available, but it seems impossible to obtain any but the briefest account of the remaining 4. These are all Russian forms and neither the titles of the publications nor the descriptions have been available.

Several species of *Plagiorchis* have been recorded from wild birds in this country by Nicoll (1923) and Baylis (1939). They are *P.* (*M.*) *cirratus* (Rud. 1802), *P.* (*M.*) *notabilis* Nicoll, 1909, *P.* (*P.*) *maculosus* (Rud. 1802), *P.* (*P.*) *elegans* (Rud. 1802), *P.* (*P.*) *permixtus* Braun, 1901, *P.* (*P.*) *triangularis* (Dies. 1850), *P.* (*P.*) *nanus* (Rud. 1802), and *P.* (*P.*) *vitellatus* (v. Linst. 1875). Only 2 of these belong to the subgenus *Multiglandularis*. The writer was able to examine specimens of some of these, together with some other species of the genus at the Natural History Museum, and would like to express her grateful thanks to Mr S. Prudhoe for his very kind help on this occasion. There is no doubt that the present species is quite different from any previously recorded in this country with, of course, the exception of Foggie's specimens from Northern Ireland. Other species of the genus have been recorded from various parts of the world: from Europe by Lühe (1909), Mühling (1896), Braun (1901, 1902), Nöller & Ullrich (1927); from America by McMullen (1937*a*, *b*), Park (1936); from Russia by Skrjabin & Massino (1925), Massino (1929) and Ström (1940 *a*, *b*); from Japan by Yamaguti (1939); from India by Mehra (1937) and from China by Kee (1940). The genus is therefore widely distributed, and the species occur in a large variety of wild birds and a few fishes, Amphibia and reptiles.

In the present instance it is extremely unlikely that the turkey is the normal host of the parasite. The probability is that the species is normally a parasite of some wild bird, and the young turkeys having been exposed to infection picked up excessively large numbers of the parasite with fatal results. In the collection at the Natural History Museum there was one prepared slide of *P.* (*P.*) *maculosus* (Rud.) found in a turkey poult in Suffolk in 1932, but no further information was available concerning it.

It is possible that a trematode in an unusual host may become modified in various ways so as to be almost unrecognizable as the original species, but it seems unlikely that precisely the same alteration should take place in Northern Ireland and Wales. Also, the specimen of *P.* (*P.*) *maculosus* from a turkey seen at the Natural History Museum showed no differences from the same species recovered from the normal bird hosts. Four other species of trematodes have been recorded from turkeys which normally occur in other bird hosts and they, too, showed no change in structure relative to the host. They are *Echinoparyphium recurvatum* (v. Linst.) in America, normally in fowl, duck and wild ducks, *Harmostomum commutatum* (Dies.) in South Europe, North Africa and Indo-China from guinea-fowl and pigeon, *Cyclocoelum mutabilis* (Zeder) usually in moorhen and *Collyriclum faba* (Bremser) usually in various passerine birds in Europe and North America.

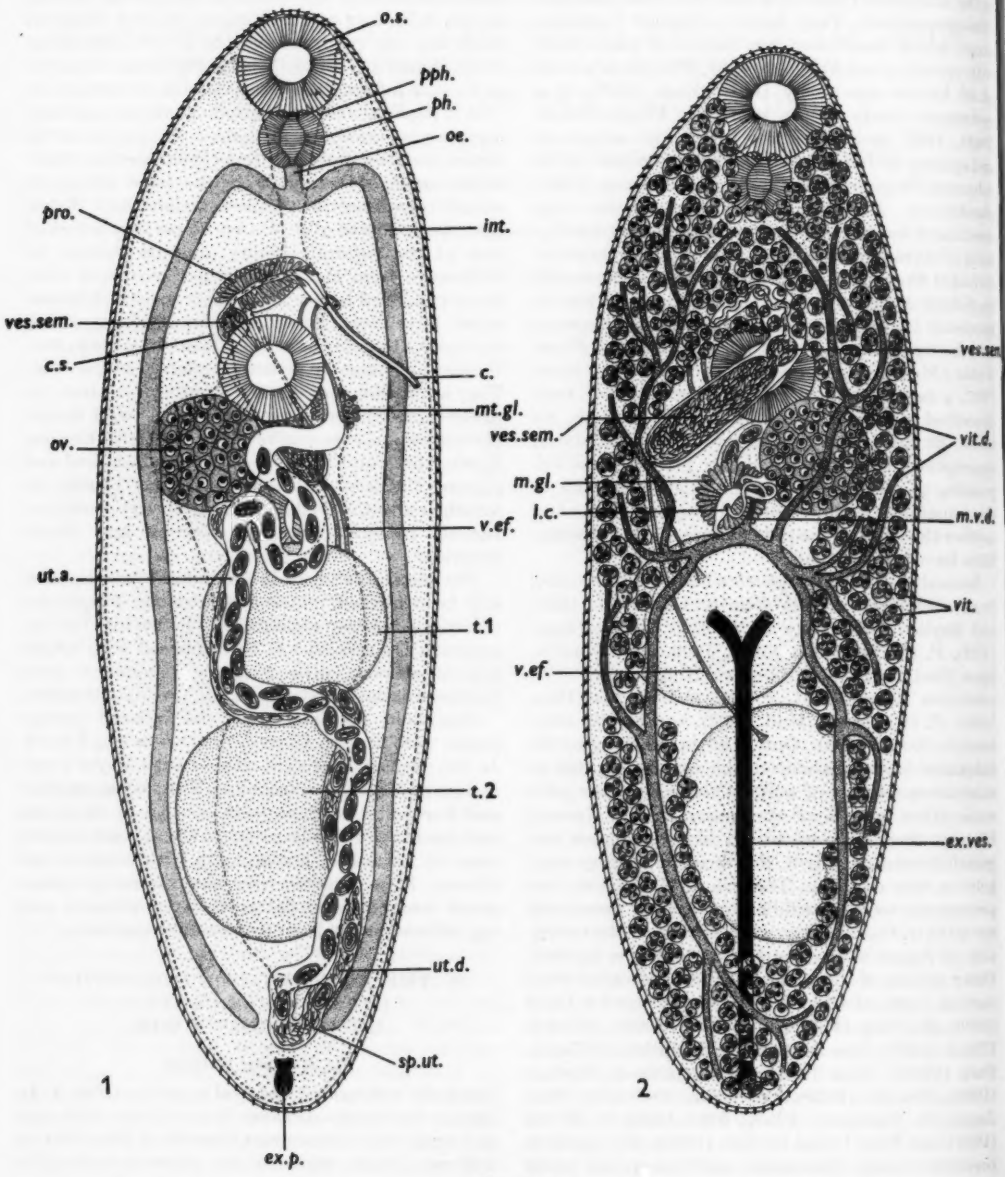
The specimens found in the present investigation will be described under a new name, *Plagiorchis* (*Multiglandularis*) *megalorchis*. Following the description the species will be compared with nearly related species, with Foggie's specimens from Northern Ireland and with *P.* (*M.*) *laricola* Skrjabin.

Specimens obtained from the infested turkey poults were fixed in Gilson's fixative or 5% formol. *In toto* preparations were stained with Meyer's carmalum or Meyer's paracarmine. Transverse, sagittal and horizontal sections were cut 5 μ in thickness and stained with Mallory's triple stain, Heidenhain's azan or Ehrlich's haematoxylin with eosin or van Gieson. Measurements were made from specimens which had not been flattened under pressure and egg measurements from a macerated specimen.

II. THE ANATOMY OF *PLAGIORCHIS* (*MULTIGLANDULARIS*) *MEGALORCHIS* N.NOM.

(1) *External features*

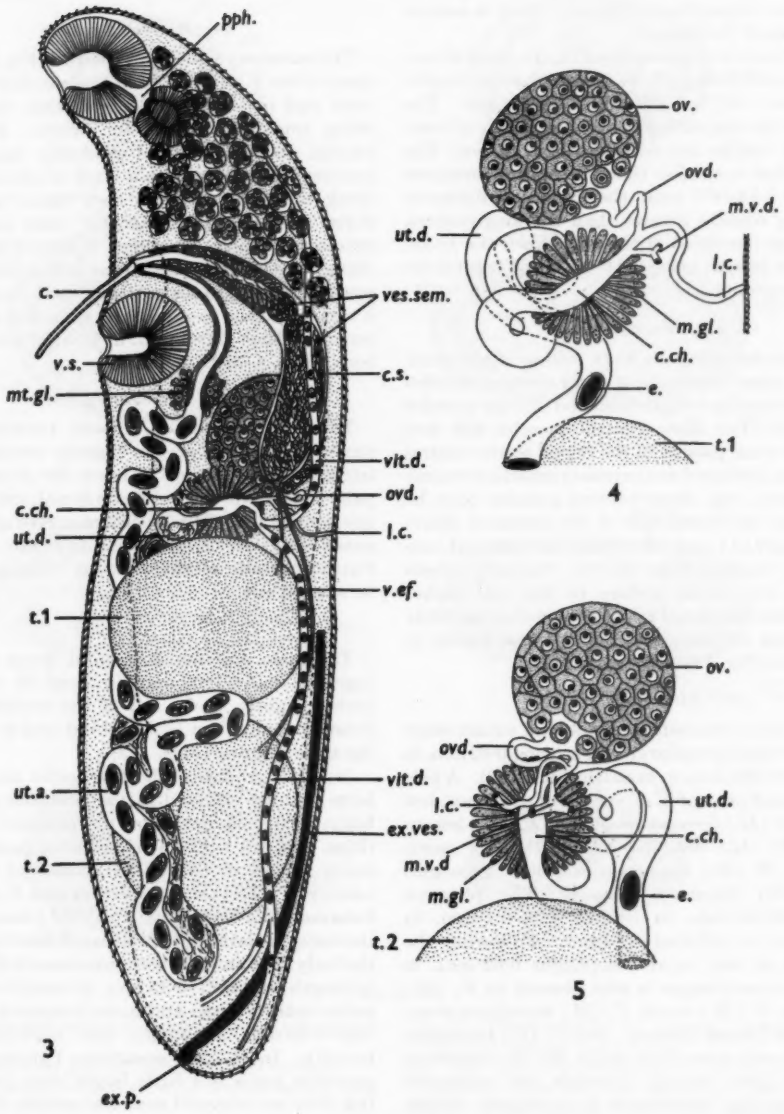
The body is elongated and oval in outline (Figs. 1–3). Mature specimens measure from 1·70 to 2·16 mm. in length with a maximum breadth of from 0·54 to 0·60 mm. in the region of the anterior testis. The body is flattened dorso-ventrally and measures 0·34 mm. in depth in the same region. The cuticle is 0·006 mm. thick and as in some other species, e.g. *P.* (*M.*) *multiglandularis* Semenov, and *P.* (*M.*) *cirratus* (Rud.), is armed over the whole surface with



Figs. 1–2.

Fig. 1. *Plagiorchis* (*M.*) *megalorchis* n.nom., ventral view, outline of vitellaria indicated by dotted line.

Fig. 2. Dorsal view to show, mainly, distribution of vitellaria and vitelline ducts.



Figs. 3–5.

Fig. 3. *P.* (*M.*) *megalorchis* n.nom., lateral view, area of vitellaria posterior to cirrus-sac indicated by dotted line.

Fig. 4. Reconstruction of female genitalia, lateral view.

Fig. 5. Reconstruction of female genitalia, dorsal view.

transverse rows of spines measuring 0·007 mm. in length. These are deeply embedded in the cuticle, traversing its whole depth and projecting only slightly from the surface, a feature which is seen in other species of the genus.

The oral sucker is subterminal on the ventral surface, its breadth being a little greater than its length. It measures 0·174–0·220 × 0·166–0·190 mm. The aperture of the oral sucker is in the majority of cases rounded in outline but occasionally it is oval. The ventral sucker is smaller than the oral, it measures 0·15–0·18 × 0·14–0·17 mm., the transverse diameter again being slightly greater than the longitudinal. It lies about one-third of the way down the body, the anterior margin being 0·44–0·50 mm. behind the anterior extremity.

(2) *Musculature*

The musculature of the body wall is of the usual trematode type, consisting of outer circular muscles followed in turn by longitudinal and oblique muscles (Figs. 6–18). The dorso-ventral muscles are well developed; some pass from the dorsal to the ventral body wall and others form extrinsic muscles for some of the organs, e.g. dorso-ventral muscles may be inserted into the dorsal side of the intestinal diverticula (Figs. 9, 11 and 12), others are inserted into the ventral sucker (Figs. 14–17). Similarly, fibres pass from the dorsal surface to the oral sucker (Fig. 18). Another set of extrinsic muscles, probably of a different origin, pass from the oral sucker to the pharynx (Fig. 18).

(3) *Alimentary canal*

The mouth in the centre of the oral sucker leads into a short broad prepharynx measuring 0·02 mm. in length and 0·065 mm. in breadth (Figs. 1–3). A prepharynx is not present in all species of the genus, but occurs in *P.* (*M.*) *ferruginum* Mehra, *P.* (*M.*) *brauni* Massino, *P.* (*M.*) *notabilis* Nicoll, *P.* (*M.*) *muris* Yamaguti, *P.* (*M.*) *maculosus motacilla* Yamaguti and *P.* (*P.*) *blumbergi* Massino. The pharynx measures 0·104 mm. in length, 0·08–0·1 mm. in breadth and is trilobed in front. Following the pharynx is a very short oesophagus 0·02 mm. in length. An oesophagus is also present in *P.* (*M.*) *ferruginum*, *P.* (*M.*) *muris*, *P.* (*M.*) *maculosus motacilla*, *P.* (*M.*) *loossi* Massino, and *P.* (*P.*) *himalayai* Mehra. Shortly after their origin the two intestinal diverticula turn slightly forwards and outwards before extending backwards to terminate within a short distance of the posterior end of the body (Fig. 1). They are broadest immediately behind the oesophagus, where they measure 0·06 mm. wide and 0·073 mm. deep, as seen in transverse section. As they proceed backwards they narrow slightly, and at about half way down the body measure 0·04 mm. wide and 0·062 mm. deep. Thereafter, to the posterior end, there is no noticeable change in size. The intestine is not easily seen in whole mounts owing to the extensive development of the vitellaria.

(4) *Excretory system*

The excretory vesicle is Y-shaped (Fig. 2). The two arms of the Y lie dorsally in the anterior half of the body and measure 0·068 × 0·029 mm. in diameter, being transversely oval in section. Behind the ventral sucker the arms gradually approach one another (Fig. 9), and at a level of about half way along the anterior testis they unite in the mid-dorsal line (Figs. 2, 10). This main stem of the excretory vesicle measures 0·145 × 0·041 mm. in diameter. It continues towards the posterior end passing dorsally to the posterior testis, behind which it turns ventrally to open by an excretory pore on the ventral surface 0·083 mm. in front of the tip of the body (Figs. 1–3, 12 and 13).

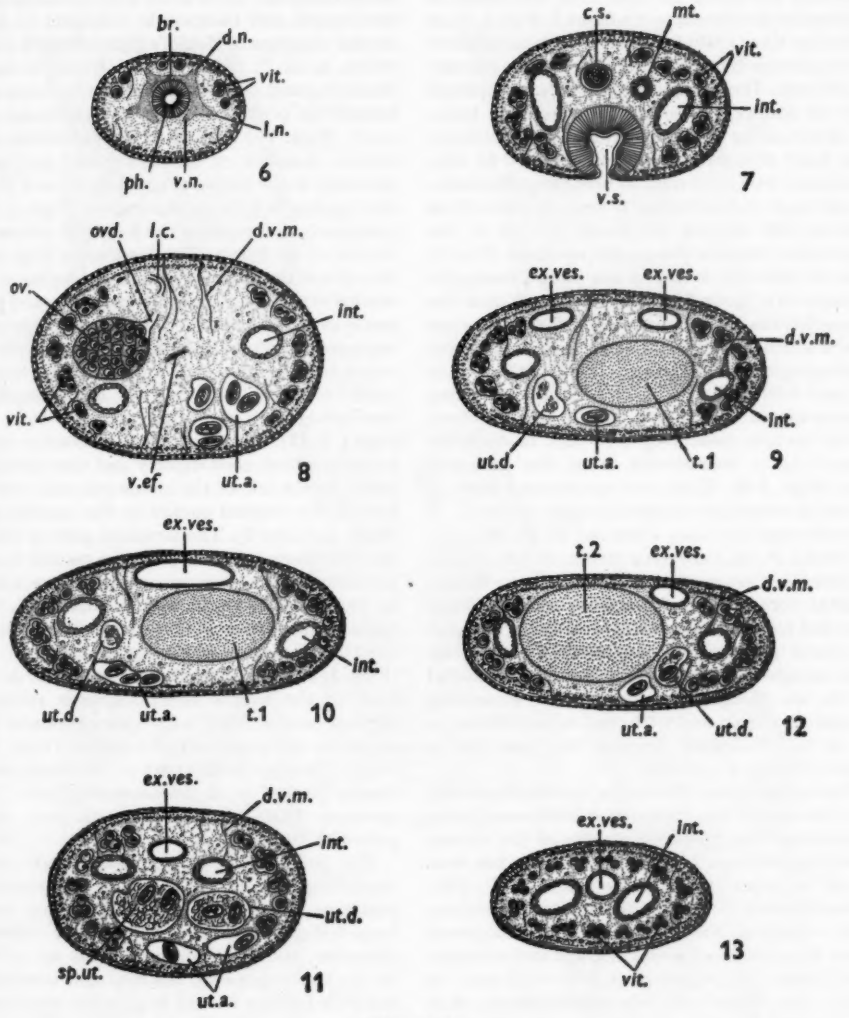
(5) *Nervous system*

The brain is situated dorsally between the oral sucker and the pharynx, slightly overlapping the latter (Fig. 6). From the brain are given off three pairs of nerves posteriorly: one dorsal, one lateral and one ventral pair. Three pairs of nerves are given off anteriorly; they lie on either side of the oral sucker. Further details of the nervous system could not be worked out.

(6) *Genitalia*

The male and female genital ducts open close together, 0·016–0·02 mm. in front of the ventral sucker slightly to the left of the middle line. The female opening is a little behind and to the left of the male opening (Figs. 1, 3).

(*a*) *Male genitalia.* The two testes are unusually large and are situated in the posterior part of the body, 0·26–0·30 mm. from the posterior extremity (Figs. 1–3 and 9–12). This posterior position of the testes occurs in two other species of the genus, namely, *P.* (*M.*) *extremus* Ström and *P.* (*M.*) *maculosus motacilla* Yamaguti. In *P.* (*M.*) *brauni* Massino the testes are large but are situated near the middle of the body. The anterior testis measures 0·27–0·37 mm. in length and 0·27–0·35 mm. in breadth. The posterior testis is larger and more elongated, measuring 0·35–0·50 mm. in length and 0·28–0·38 mm. in breadth. In *P.* (*M.*) *mutationis* Panowa, also, the posterior testis is a little larger than the anterior, but they are situated near the middle of the body. Owing to the large size of the testes their obliquity is not very well marked, but the anterior testis is a little to the left and the posterior a little to the right of the median line; the intertesticular space is narrow (Figs. 1, 2). Both vasa efferentia arise antero-dorsally from their respective testes and run forwards in the dorsal half of the body to join just



Figs. 6–13.

Fig. 6. Transverse section through pharynx to show part of brain.

Fig. 7. Transverse section through ventral sucker.

Fig. 8. Transverse section showing oviduct arising from ovary.

Fig. 9. Transverse section showing anterior testis and two excretory ducts.

Fig. 10. Transverse section showing anterior testis and union of two excretory ducts.

Fig. 11. Transverse section of intertesticular region showing descending and ascending limbs of uterus.

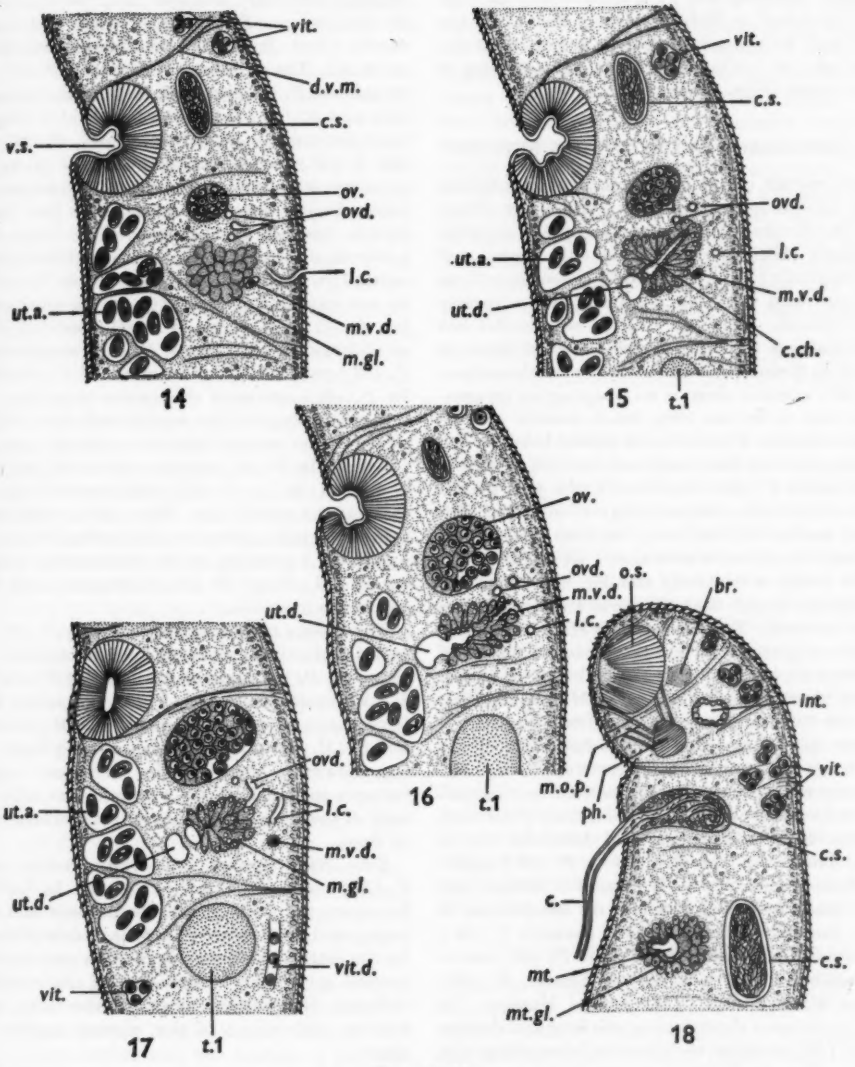
Fig. 12. Transverse section showing posterior testis and stem of excretory vesicle.

Fig. 13. Transverse section through post-testicular region showing vitellaria encircling body.

as they enter the cirrus-sac (Figs. 2, 3). In *in toto* preparations the cirrus-sac seen from the dorsal or ventral surface has the shape of a figure 7 (Figs. 1, 2), as described by Kee (1940) for *P.* (*M.*) *multiglandularis* Semenov, though the disposition of the organ is somewhat different. The stem of the 7 is obliquely placed dorsally to the ventral sucker and extends backwards almost as far as the posterior end of the ovary. It is a large structure, measuring 0·60–0·67 mm. in length and 0·09–0·10 mm. in breadth proximally. In lateral view the cirrus-sac is seen to curve from the dorsal side around the dorsal border of the ventral sucker towards the genital aperture (Fig. 3). The curve, however, is not in one plane; hence the appearance of a figure 7 in surface view. Within the cirrus-sac the vas deferens enlarges to form a vesicula seminalis which is divided into two parts, a proximal cylindrical portion measuring 0·21–0·27 mm. in length and 0·06–0·07 mm. in breadth, almost filling the cirrus-sac in this region, and a smaller distal spherical portion measuring 0·05 mm. in diameter separated by a constriction from the proximal portion (Figs. 1–3). These two constituent parts of the vesicula seminalis occupy the stem of the 7. A similar division has been observed in *P.* (*M.*) *cirratus* (Rud.), *P.* (*M.*) *extremus* Ström, *P.* (*M.*) *maculosus motacilla* Yamaguti, and *P.* (*P.*) *upupae* Ström. The distal portion of the cirrus-sac narrows fairly rapidly and contains the pars prostatica surrounded by prostatic gland cells, the ductus ejaculatorius and the invaginated cirrus (Fig. 2). The evaginated cirrus is an elongated sinuous tube measuring 0·177 mm. in length and 0·01 mm. in breadth at its base. It tapers slightly towards the apex and is unarmed (Figs. 1, 3 and 18).

(*b*) *Female genitalia*. The ovary lies slightly to the right of the middle line, its anterior border extending a little beyond the posterior margin of the ventral sucker (Figs. 1–3 and 14–17). This overlap has been observed in some other species, namely, *P.* (*M.*) *skrjabini* Massino, *P.* (*M.*) *multiglandularis* Semenov, *P.* (*M.*) *notabilis* Nicoll, and *P.* (*P.*) *uhlwormii* Massino. It is almost spherical in shape and measures 0·20–0·22 mm. in length and 0·18–0·22 mm. in breadth. It is larger than the ventral sucker, as in *P.* (*M.*) *brauni* Massino, *P.* (*M.*) *skrjabini* Massino, *P.* (*M.*) *extremus* Ström and some others. The oviduct, 0·01 mm. in diameter, arises from the inner posterior border of the ovary towards the dorsal side (Figs. 2–5 and 8). It runs obliquely posteriorly, coiling slightly, towards the centre of the body. Opening into the oviduct dorsally is Laurer's canal, 0·008 mm. in diameter. Laurer's canal takes a slightly sinuous course postero-dorsally to open to the exterior almost in the middle line a short distance behind the ovary (Figs. 2–5, 8 and 14–17). The median yolk duct opens into the oviduct a short distance beyond the opening of Laurer's canal (Figs. 2–5 and 16). The oviduct turns ventrally where these two ducts enter and continues obliquely downwards and backwards enlarging to form the central chamber of Mehlis's gland (Figs. 2–5 and 15), which, as in *P.* (*M.*) *cirratus* (Rud.), is elongated. Mehlis's gland occupies the space between and just behind the proximal end of the cirrus-sac and the ovary (Figs. 1–5 and 14–17). Continuous with the central chamber of Mehlis's gland and emerging ventrally is the uterus (Figs. 3–5, 16 and 17), which after coiling a little in this region (Figs. 4, 5) turns posteriorly and pursues the S-shaped course characteristic of the genus. The descending limb passes to the right of the anterior testis overlapping it slightly, crosses ventrally between the anterior and posterior testes to continue down the left side of the posterior testis into the post-testicular region where it loops round on itself forming the ascending limb which passes forwards following the same course as the descending limb and lying ventrally to it (Figs. 1, 3 and 9–12). In front of the anterior testis the ascending limb coils slightly and then proceeds forwards to the left of the cirrus-sac and over the left side of the ventral sucker to the genital aperture (Figs. 1, 3 and 7). The terminal part of the uterus, the metraterm, extends from the genital aperture to just behind the posterior margin of the ventral sucker. It measures 0·05–0·09 mm. in diameter, is thick-walled and surrounded proximally by numerous unicellular glands which apparently open into it (Figs. 1, 3, 7 and 18). The whole of the descending limb of the uterus functions as a receptaculum seminis and is filled with sperms almost from its origin to the post-testicular region (Figs. 1, 3 and 9–12). The eggs in this part of the uterus are not as closely packed as in the ascending limb. The eggs measure 0·030–0·033 × 0·021–0·023 mm. and have yellowish brown shells.

The yolk glands are extremely well developed, extending from the level of the oral sucker to the posterior extremity (Figs. 1–3). They consist of large follicles measuring up to 0·037–0·058 mm. in diameter, and in exceptional cases up to 0·09 mm. In the region between the oral and ventral suckers the yolk follicles extend in a broad band across the dorsal surface (Fig. 2). They do not join ventrally but approach the middle line (Fig. 1). In the post-testicular region they are continuous dorsally and ventrally, forming a ring around the body which is interrupted ventrally by the excretory pore (Figs. 1, 2 and 13). On either side of the body the yolk glands encroach slightly, both dorsally and ventrally, over the lateral margins of the ovary and testes and in the intervals between these organs (Figs. 1, 2, 8 and 10). Ducts from the yolk follicles join up in groups, and these eventually open into the lateral yolk ducts running down each side of the body on the dorsal side (Fig. 2). These are branched; the



Figs. 14–18.

Fig. 14. Sagittal section showing opening of Laurer's canal to exterior.

Fig. 15. Sagittal section showing central chamber of Mehlis's gland.

Fig. 16. Sagittal section showing yolk duct entering oviduct.

Fig. 17. Sagittal section showing Laurer's canal entering oviduct.

Fig. 18. Sagittal section showing evaginated cirrus, cirrus-sac and metraterm with glands.

branching on either side is not symmetrical but the same general pattern is maintained. The two lateral yolk ducts communicate by a transverse yolk duct which lies dorsal to Mehlis's gland. From this the median yolk duct arises and passes antero-ventrally to open into the oviduct just beyond the opening of Laurer's canal (Figs. 2–5 and 16).

III. COMPARISON WITH OTHER SPECIES

The two species of the subgenus *Multiglandularis* already known to occur in this country are *Plagiorchis* (*M.*) *cirratus* (Rud.), recorded by Nicoll (1923) and Baylis (1939) from various wild birds, and *P.* (*M.*) *notabilis* Nicoll, recorded by the same authors again from wild birds but not from such a variety of host species. Although *P.* (*M.*) *megalorchis* has certain features in common with both of these, it differs from them in certain fundamental characters. In *P.* (*M.*) *cirratus* there is no prepharynx or oesophagus, the testes are very much smaller and lie farther forwards. The cirrus-sac passes between the ventral sucker and the ovary and does not extend as far back as in *P.* (*M.*) *megalorchis*, the yolk glands are not as extensive, commencing not on a level with the oral sucker but half way between the ventral sucker and the anterior extremity. In *P.* (*M.*) *notabilis* the ovary is relatively smaller. The testes are only slightly larger than the oral sucker and are farther forwards. The cirrus-sac is short and stout and does not extend beyond the posterior border of the ventral sucker, the yolk glands begin on a level with the pharynx and, unlike *P.* (*M.*) *megalorchis*, only a few follicles pass across the body dorsally.

Of the species recorded from other parts of the world, there are again some points of similarity, but the differences are such that the present species cannot be included in any one of which descriptions are available. Some of the points of difference are as follows. There is no prepharynx in *P.* (*M.*) *multiglandularis* Semenov, *P.* (*M.*) *casarcii* Mehra, and *P.* (*M.*) *mutationis* Panowa and no oesophagus in *P.* (*M.*) *multiglandularis*, *P.* (*M.*) *casarcii*, *P.* (*M.*) *micromaculosus* Skrjabin & Massino, *P.* (*M.*) *skrjabini* Massino, *P.* (*M.*) *eutamiatus* Schulz, *P.* (*M.*) *extremus* Ström and *P.* (*M.*) *brauni* Massino. In *P.* (*M.*) *mutationis* the oesophagus is long and slender and in *P.* (*M.*) *skrjabini* the pharynx is larger than the ventral sucker. In *P.* (*M.*) *arcuatus* Ström the caeca are in contact behind the testes. The ovary is smaller than the ventral sucker in *P.* (*M.*) *arcuatus*, *P.* (*M.*) *micromaculosus*, *P.* (*M.*) *micronotabilis* Semenov, *P.* (*M.*) *muris* Tanabe, *P.* (*M.*) *melanderi* Semenov, *P.* (*M.*) *multiglandularis*, *P.* (*M.*) *potanini* Skrjabin, *P.* (*M.*) *mentulatus* (Rud.), and *P.* (*M.*) *arvicolae* Schulz & Skworzow. The ovary does not overlap the ventral sucker in *P.* (*M.*) *micromaculosus* and *P.* (*M.*) *casarcii*, and is median and behind the ventral sucker in *P.* (*M.*) *russii* Mehra, *P.* (*M.*) *extremus* and *P.* (*M.*) *massino* Petrov & Tichonov. In *P.* (*M.*) *loossi* Massino the ovary is larger than the testes, and in *P.* (*M.*) *asperus* Stossich the ovary and testes are deeply lobed. In *P.* (*M.*) *multiglandularis* the testes are small. The cirrus-sac is shorter in *P.* (*M.*) *micromaculosus*, *P.* (*M.*) *casarcii*, *P.* (*M.*) *maculosus motacilla* and *P.* (*M.*) *loossi* Massino, and is longer and more extensive in *P.* (*M.*) *skrjabini*, *P.* (*M.*) *brauni* and *P.* (*M.*) *ferruginum* Mehra. There are only four species in which the vesicula seminalis is described as being divided into two parts. The fact that this feature has not been mentioned in other species, however, does not necessarily mean that it does not exist. The vitellaria are less extensive, that is, they do not extend forwards beyond the pharynx if as far, in *P.* (*M.*) *muris*, *P.* (*M*) *potanini*, *P.* (*M.*) *mentulatus*, *P.* (*M.*) *loossi*, *P.* (*M.*) *micromaculosus*, *P.* (*M.*) *russii*, *P.* (*M.*) *casarcii* and *P.* (*M.*) *brauni*. In *P.* (*M.*) *arcuatus* the uterus does not extend posteriorly beyond the testes, and the suckers are equal or the ventral sucker is slightly larger than the oral. In *P.* (*M.*) *micronotabilis* the genital pore is median, and in *P.* (*M.*) *melanderi* it is far to the right of the middle line. There are species in which spines are not present on the surface of the body, e.g. *P.* (*M.*) *extremus*, or are present only anteriorly, e.g. *P.* (*M.*) *loossi*, *P.* (*M.*) *ferruginum* and *P.* (*M.*) *maculosus motacilla*.

The species most nearly resembling *P.* (*M.*) *megalorchis* in the distribution of the vitellaria are *P.* (*M.*) *arcuatus*, *P.* (*M.*) *micronotabilis*, *P.* (*M.*) *melanderi*, *P.* (*M.*) *multiglandularis*, *P.* (*M.*) *skrjabini*, *P.* (*M.*) *ferruginum*, *P.* (*M.*) *extremus*, *P.* (*M.*) *eutamiatus* and *P.* (*M.*) *mutationis*. As has already been pointed out, however, there are in each case important features in which *P.* (*M.*) *megalorchis* differs from each of these and which prevent its inclusion in any of them.

Two other species have large testes, namely, *P.* (*M.*) *russii* and *P.* (*M.*) *extremus*. In the former, however, the yolk glands begin near the ventral sucker and the testes are near the middle of the body. In the latter, the ovary is median and behind the ventral sucker, the disposition of the cirrus-sac is different, there are no spines on the body, and the relative dimensions of the various organs do not agree.

The following species cannot be compared with *P.* (*M.*) *megalorchis* as no descriptions are available of them: *P.* (*M.*) *rudolphi* Massino ?, *P.* (*M.*) *schuschovi* Issaitschikov, and *P.* (*M.*) *svetlakovi* Issaitschikov.

There is no doubt that the species described in this paper is the same as that identified provisionally by Foggie (1937) as *P. laricola* Skrjabin. As already stated, the writer has examined some of Foggie's material, made measurements of the various organs of the body and has found them to agree in every

respect. Foggie gave a very brief description of the worm and included a figure. He stated that the ovary is to the left, whereas in the present specimens and those of Foggie examined by the writer the ovary is to the right of the middle line.

It is doubtful if this species is *P. laricola*. From the few facts known about the latter, it is impossible to identify the present species as such, especially if one takes into consideration the other species described since Massino (1929) compiled his key. The three facts known about *P. laricola* are (1) the ovary is larger than the ventral sucker, (2) the vitellaria are on a level with or in front of the forking of the gut and join in the middle line anteriorly, and (3) the cirrus-sac does not extend beyond the posterior margin of the ovary. There is no mention of the extent of the junction of the vitellaria, whether it is a few follicles or a broad band which extends across the middle line. Schulz & Skworzow (1931), however, place it in the subgenus *Multiglandularis*.

The ovary is larger than the ventral sucker in several species, namely, *Plagiorchis* (*M.*) *brauni*, *P.* (*M.*) *loossi*, *P.* (*M.*) *skrjabini*, *P.* (*M.*) *cirratus*, *P.* (*M.*) *extremus*, *P.* (*M.*) *casarcii* and *P.* (*M.*) *maculosus motacilla*. The last four of these are not included in Massino's key, the last three not being known at the time. The vitellaria are on a level with or in front of the forking of the gut and join anteriorly either in a broad band or by isolated follicles in a large number of species, the majority of which are not included in Massino's key. The cirrus-sac does not extend beyond the ovary in *P.* (*M.*) *potanini*, *P.* (*M.*) *brauni*, *P.* (*M.*) *loossi*, *P.* (*M.*) *cirratus*, *P.* (*M.*) *extremus*, *P.* (*M.*) *casarcii*, *P.* (*M.*) *notabilis* and *P.* (*M.*) *maculosus motacilla*.

There are therefore several species which resemble *P. laricola* in one, two or all three characters. Those resembling it in all three are *P.* (*M.*) *notabilis*, *P.* (*M.*) *extremus* and *P.* (*M.*) *maculosus motacilla*. None of these is included in Massino's key; the last two were described after 1929. These species are quite different from one another in other anatomical features and are also different from *P.* (*M.*) *megalorchis*. Massino makes no mention of such diagnostic features for *P. laricola* as the relative dimensions of the parts of the body, the position of the ovary, the presence or absence of prepharynx or oesophagus, the size and position of the testes, and the details of the disposition and structure of the cirrus-sac. It can therefore be seen that it is impossible to identify the present species as *P. laricola* until a description of the latter can be obtained, and this seems extremely difficult as efforts have been made both in this country and the United States.

The name proposed for this species, therefore, is *Plagiorchis* (*Multiglandularis*) *megalorchis* n.nom., and it is synonymous with that described by Foggie (1937) as *P. laricola* Skrjabin.

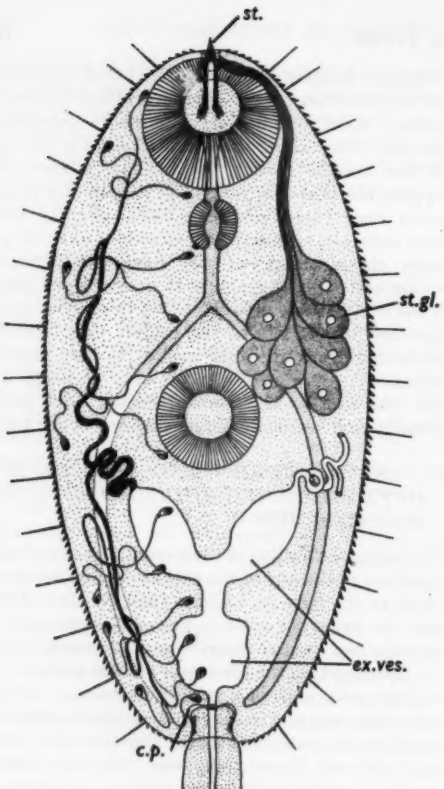
Specific diagnosis. Cuticle armed with spines over the whole surface; oral sucker larger than the ventral sucker; short prepharynx and short oesophagus present; ovary larger than, and situated to the right of, the ventral sucker, its anterior border overlapping the posterior margin of the ventral sucker; testes very large, posterior testes slightly larger than anterior, situated near posterior extremity of body; cirrus-sac curved over dorsal side of ventral sucker and extending almost to the posterior margin of the ovary; vesicula seminalis in two parts; genital aperture to the left of the middle line; vitellaria extending from the oral sucker to the posterior extremity, joining in a broad band dorsally from the pharynx to the ventral sucker and joining dorsally and ventrally in the post-testicular region.

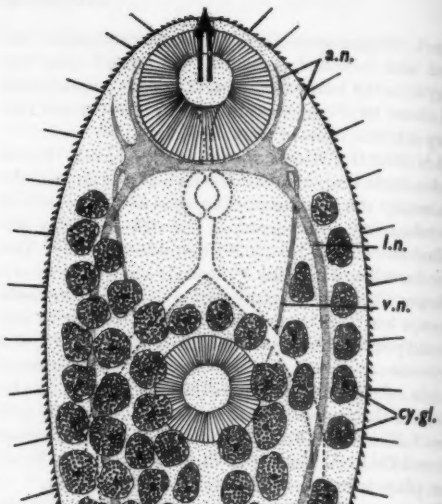
IV. LARVAL STAGES AND LIFE HISTORY OF *PLAGIORCHIS* (*MULTIGLANDULARIS*) *MEGALORCHIS*

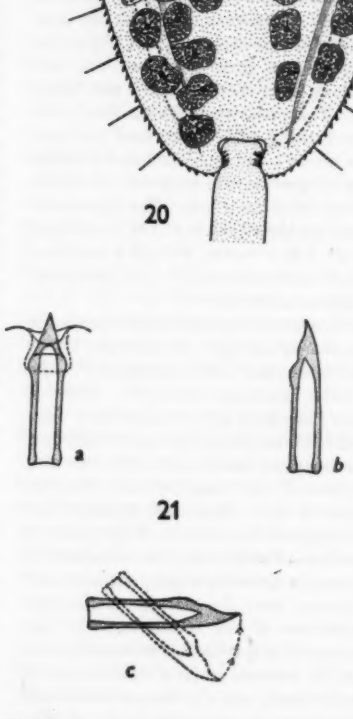
Following the finding of the trematodes in such large numbers in the gut of the turkey poults, a visit was made to the farm on 15 June 1950, by Mr F. Jordan and the writer, in order to find, if possible, how the turkeys had become infested and to obtain material for investigating the life history of the parasite. The turkeys were kept in a yard in one corner of which was a large wet and muddy area containing numerous small pools swarming with Tubifex and various insect larvae. These pools were the only source of drinking water for the young turkeys. No gastropod molluscs were found in them at the time of the visit, but in a field adjoining the yard and to which the birds had access was a stream in which were large numbers of *Lymnaea pereger* and some *Ancylus fluviatilis*. Specimens of all these were brought back to the laboratory for examination in the hope that the larval stages of *Plagiorchis* (*Multiglandularis*) *megalorchis* could be found. Further visits to collect additional material were made at a later date by Mr Jordan, to whom the writer is most grateful.

V. FIRST INTERMEDIATE HOST

No larval trematodes were found in the specimens of *Ancylus fluviatilis*. Large numbers of *Lymnaea pereger* were kept in an aquarium in the laboratory, and isolated singly in small glass vessels to find if any cercariae emerged from them. Of 140 examined, 8 or 5·7 % were parasitized by *Cercaria monostomi* which was obviously not the species concerned, 50 or 35·7 %, a relatively large proportion, were infected with a xiphidiocercaria and the remaining 82 or 58·5 % were free from parasites. The xiphidiocercariae were found to belong to the Polyadena group, Cort (1915), of the Cercariae Armatae Lühe (1909). According to Cort the members of this group belong to the subfamily Plagiorchiinae of the family







Figs. 19–21.

Fig. 19. *Cercaria P.* (*M.*) *megalorchis* n.nom., dorsal view, showing stylet glands on right and excretory system on left.

Fig. 20. *Cercaria P.* (*M.*) *megalorchis* n.nom. showing distribution of dorsal cystogenous glands and nervous system.

Fig. 21. (*a*) Stylet, dorsal view. (*b*) Stylet, lateral view. (*c*) Diagram to illustrate arc through which stylet moves during process of penetration.

Plagiorchiidae and have the following characters. They develop in elongated sac-shaped sporocysts; the tail is slender and shorter than the body except when very much extended; the acetabulum is behind the middle of the body and of a diameter almost equal to or smaller than that of the oral sucker; the stylet measures about 0·03 mm. in length, is six times as long as broad with a thickening one-third of the distance from the point to the base; there are six or more stylet glands on each side; the excretory vesicle is bicornuate and the main excretory tube divided into an anterior and posterior branch receiving ducts from three groups of three flame cells each; the flame cell formula is

$$2[(3+3+3)+(3+3+3)]=36;$$

a very short prepharynx and small pharynx present; the oesophagus, when developed, is of short or medium length; the intestinal caeca reach the posterior end of the body.

According to McMullen (1937*a*) the life histories of seven members of the Polyadena group of xiphidiocercariae are known. This is a very small percentage, as there are 67 known species in the genus *Plagiorchis* alone, and there are 60 known genera in the family Plagiorchiidae. Of the species of *Plagiorchis* infesting birds, the life histories of three have been demonstrated experimentally, namely, *P.* (*P.*) *maculosus* (Rud.) by Nöller & Ullrich (1927), *P.* (*M.*) *muris* Tanabe and *P.* (*P.*) *proximus* by McMullen (1937*b*).

The xiphidiocercaria found in the present instance was proved experimentally, as will be shown later, to be the larval stage of the trematode *P.* (*M.*) *megalorchis* found in the turkey poults. Many cercariae of this group have been described by various writers, namely, Lühe (1909), Cort (1915), Sewell (1922), Dubois (1929), Wesenberg-Lund (1931) and others, but the present form cannot be identified with any of them. A description of it is therefore given below. The anatomy has been worked out from living cercariae which had emerged naturally from the snail host and from sections of the digestive gland of infected molluscs. For this latter purpose the digestive gland was fixed in Gilson's fixative, sections were cut 5 μ in thickness, and stained with Heidenhain's azan, Mallory's triple stain or Ehrlich's haematoxylin with Orange G.

VI. CERCARIA *PLAGIORCHIS* (*MULTIGLANDULARIS*) *MEGALORCHIS*

Measurements

Body: length, 0·258 mm. (at rest), 0·323 mm. (extended); breadth, 0·135 mm. (at rest), 0·052 mm. (extended).

Tail: length, 0·156–0·187 mm., 0·083 mm. (contracted); breadth near base 0·029 mm.

Stylet: length, 0·031–0·033 mm.; breadth of shaft, 0·008 mm.

Oral sucker: diameter, 0·062–0·064 mm.

Ventral sucker: 0·041 mm.

Pharynx: diameter, 0·20–0·22 mm.

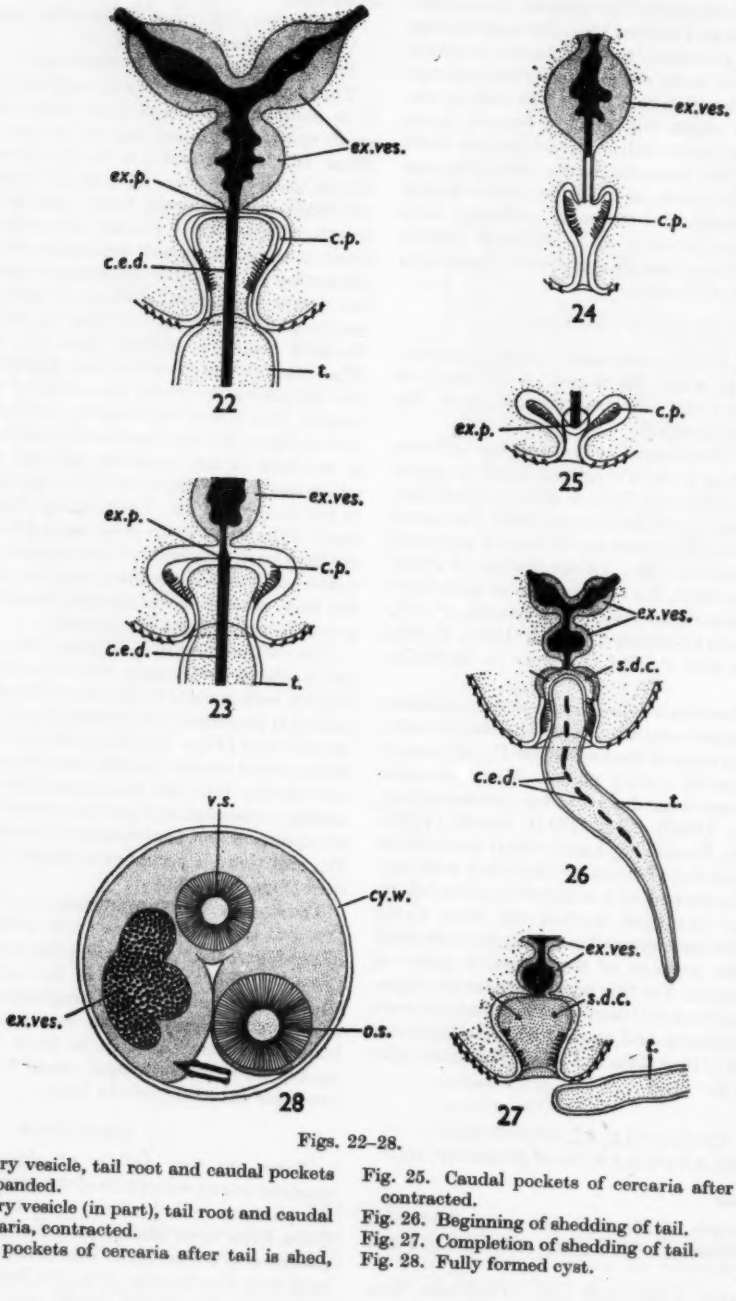
The body is greyish in colour and very contractile. It is completely covered with transverse rows of small spines decreasing slightly in size posteriorly (Figs. 19, 20). In addition to the spines there are fifteen transverse rows of sensory hairs encircling the body and arranged fairly regularly along its length. The stylet is placed horizontally with its point, situated in a small depression on the surface, projecting just beyond the anterior extremity. On the dorsal and lateral surfaces of the stylet about one-third of its length from the tip is a projecting shoulder which is absent from the ventral side (Fig. 21*a*, *b*). The shoulder rests against the base of the depression in which the apex of the stylet is lodged. In a lateral view the tip of the stylet can be seen to curve slightly in a dorsal direction. Attached to the base of the stylet are delicate muscle fibres which are continuous with the longitudinal muscles of the body (Fig. 19). These bring about the movement of the stylet when penetrating the second intermediate host. The body of the cercaria itself is fairly muscular; fine longitudinal muscles can be seen in the body wall in living specimens, and below them a double series of oblique muscles.

The root of the tail is enclosed in a depression at the posterior extremity, the opening of which is slightly subterminal on the ventral surface. A caudal pocket is present in this depression on either side of the tail root (Figs. 19, 20 and 22–25). The cuticular lining of the caudal pockets is thickened and frayed out distally into fine processes. The extent of the caudal depression and pockets varies with the state of expansion and contraction of the body (Figs. 22, 23), and there is a change in shape after the tail is shed (Figs. 24, 25).

The oral sucker is subterminal on the ventral surface and the ventral sucker just behind the middle of the body, the former being larger than the latter (Figs. 19, 20). The mouth in the centre of the oral sucker leads into a short prepharynx followed by a muscular pharynx and an oesophagus which bifurcates some distance in front of the ventral sucker into two intestinal caeca which reach the posterior extremity of the body.

(1) *Stylet glands*

There are two groups of eight stylet glands situated one on either side of the oesophagus between the pharynx and the ventral sucker (Fig. 19). The ducts from these glands pass forwards to open by a series of pores on either side of the stylet at a point level with the shoulder (Fig. 19). Transverse sections show that the stylet glands are dorsally placed



Figs. 22–28.

Fig. 22. Excretory vesicle, tail root and caudal pockets of cercaria, expanded.
Fig. 23. Excretory vesicle (in part), tail root and caudal pockets of cercaria, contracted.
Fig. 24. Caudal pockets of cercaria after tail is shed, expanded.
Fig. 25. Caudal pockets of cercaria after tail is shed, contracted.
Fig. 26. Beginning of shedding of tail.
Fig. 27. Completion of shedding of tail.
Fig. 28. Fully formed cyst.

(Figs. 31, 32). They contain a finely granular cytoplasm and a rounded nucleus containing one macronucleolus and two micronucleoli. In living specimens the cytoplasm is greyish in colour and the nuclei clear. They are easily distinguished from the cystogenous gland cells.

(2) *Cystogenous glands*

The cystogenous gland cells are situated dorsally and laterally. They are more abundant in the posterior half of the body and extend forwards on either side in the region between the oral and ventral suckers. The anterior mid-dorsal region is devoid of glands (Figs. 20 and 31–36). The cystogenous gland cells are faintly yellow in living specimens and are packed with small granules staining readily with Orange G. Each cell is somewhat irregular in outline and contains a rounded nucleus.

(3) *Ventral glands*

There are eight large ventral gland cells arranged symmetrically to right and left of the middle line, two pairs in front of and two pairs behind the ventral sucker (Figs. 29–36). They are slightly irregular in outline, with a large nucleus containing one macronucleolus and two micronucleoli. In transverse section they can be seen to occupy about half the depth of the body.

(4) *Dorsal glands*

These are small cells situated dorsally and laterally just below the body wall (Figs. 29, 31–34 and 36). They are most abundant anteriorly and posteriorly around the root of the tail. They are irregular in shape and each is provided with a rounded nucleus.

The tail is also filled with glandular cells.

(5) *Excretory system*

The excretory vesicle is Y-shaped and extends from the root of the tail to the posterior border of the ventral sucker (Figs. 19, 22). It is lined by a layer of epithelial cells each with a large nucleus causing the cell to bulge into the lumen of the vesicle (Fig. 37). From each arm of the excretory vesicle arises the main lateral excretory vessel which passes anterolaterally, for a short distance being somewhat coiled (Fig. 19). This divides into an anterior and posterior collecting vessel which extend one anteriorly and the other posteriorly along the length of the body. Both of these vessels receive ducts from three groups each of three flame cells, the flame-cell formula being $2[(3+3+3)+(3+3+3)]=36$. In each group of three, two flame cells are internally placed and one externally, an arrangement similar to that in *Cercaria helvetica* VII Dubois (1929). The prototype of this group of cercariae, namely, *C. polyadena* Cort has a flame-cell formula of

$$2[(3+3+3)+(3+3+2)]=34,$$

there being only two flame cells in the posterior group. The tail contains a single caudal excretory duct which passes forwards and opens by the main excretory pore situated on the ventral side at the junction of the body and the tail (Fig. 19).

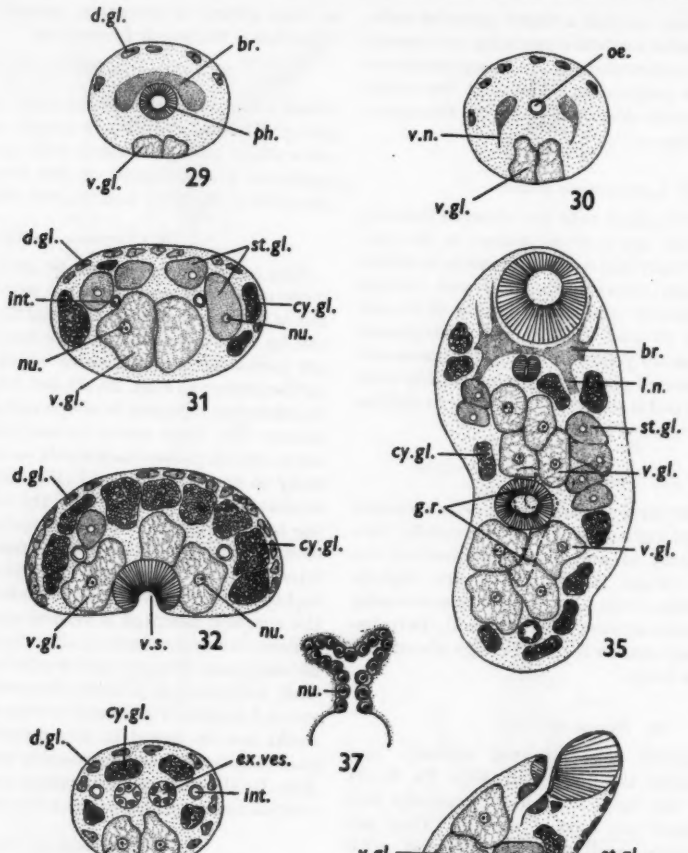
(6) *Nervous system*

The cerebral ganglia can be seen in living specimens as a clear area on either side of the pharynx, but in stained-sectioned material the nervous system can be seen more clearly. The two cerebral ganglia are joined by a commissure which passes dorsally to the pharynx (Figs. 20, 29 and 35). Each ganglion is somewhat irregular in shape and gives rise to four nerves. The main nerve on each side is the lateral nerve which passes backwards on either side of the body to the posterior end (Fig. 20). There are two anterior nerves on either side; the inner curves round the lateral margin of the oral sucker and the outer diverges slightly from this to pass to the anterolateral margin. Between the outer anterior nerve and the lateral nerve on either side there arises from the cerebral ganglion a ventral nerve. The ventral nerves turn ventrally soon after their origin (Figs. 20, 30) and pass obliquely backwards to the ventral body wall, terminating a short distance in front of the ventral sucker. The dorsal nerves seen in the adult could not be found in the cercaria nor the third pair of anterior nerves; possibly they develop later. Also, by the time the adult stage is reached, it is the ventral nerves which are the best developed.

(7) *Genital system*

Rudiments of the genitalia are present in the neighbourhood of the ventral sucker. These rudiments consist of a mass of small cells beginning in front of the ventral sucker where the genital pore will develop, passing backwards dorsally to the sucker and continuing behind it to terminate nearly half way between the ventral sucker and the posterior extremity (Figs. 35, 36). Irregular blunt projections are given off from this strand of cells on either side.

Cercaria P. (*M.*) *megalorchis* resembles fairly closely some other species of xiphidiocercariae of the Polyadena group, e.g. there are eight pairs of stylet glands in *C. helvetica* IV and XXX Dubois (1929), *C. isocotylea* Cort (1915), *C. P.* (*M.*) *muris* McMullen (1937*b*), *C. P.* (*P.*) *microcanthus* (McMullen, 1937*b*) and *C. cambrensis* III Rees (1932). The present form differs from each of these in various particulars, e.g. the dimensions of the body and tail, the relative sizes of the oral and ventral suckers, the diameter of the cyst and the host species. It seems not to have been described previously.



Figs. 29–37.

Fig. 29. Transverse section through pharynx of cercaria showing brain.
Fig. 30. Transverse section through cercaria showing ventral nerves and ventral glands.
Fig. 31. Transverse section through preacetabular region of cercaria.
Fig. 32. Transverse section through ventral sucker.
Fig. 33. Transverse section through anterior part of excretory vesicle.
Fig. 34. Transverse section through posterior part of excretory vesicle.
Fig. 35. Horizontal section through cercaria showing ventral glands, part of nervous system and rudiments of genitalia.
Fig. 36. Sagittal section through cercaria showing distribution of glands and rudiments of genitalia.
Fig. 37. Excretory vesicle showing lining of cells.

Very few cercariae emerge from the snail host during the daytime. They begin to appear in the water in the evening, and large numbers emerge at night and during the early morning. After emergence the survival time of the larvae is about 24 hr., but they are most active during the first half of that period. Later, they become gradually more sluggish, active swimming being replaced by a creeping movement along the floor of the vessel containing them.

VII. SPOROCYST

The cercariae develop in elongated sac-shaped sporocysts measuring up to 2 mm. in length and 0·28 mm. in breadth. They are of a pale yellow or orange colour. The wall consists of two layers of cells. In young sporocysts these layers are relatively thick and consist of well-developed cells with large nuclei. The cells of the internal layer are slightly larger than those of the external (Fig. 38). Between the two layers are some delicate muscle fibres. In very young sporocysts another layer of cells is present outside the external layer, the 'primitive epithelium' described by Dubois (1929) for the sporocyst of *C. helvetica* VII. This is a very delicate layer of flattened cells, the nuclei of which protrude slightly on the outer surface. It is not apparent in older sporocysts. As the sporocyst increases in size and as the cercariae develop within it, the wall becomes very much thinner, the two layers of cells becoming much flattened (Figs. 39, 40). There was no sign of a birth pore, though one has been described in the sporocysts of some members of this group.

VIII. EFFECT OF THE PARASITES ON THE TISSUES OF THE FIRST INTERMEDIATE HOST

Several workers have examined the effect of parasites on the tissues of the digestive gland of the molluscan host, namely, Faust (1917, 1920), Langeron (1920), Dubois (1929) and Rees (1931). In the present instance the histological changes are similar to those previously described.

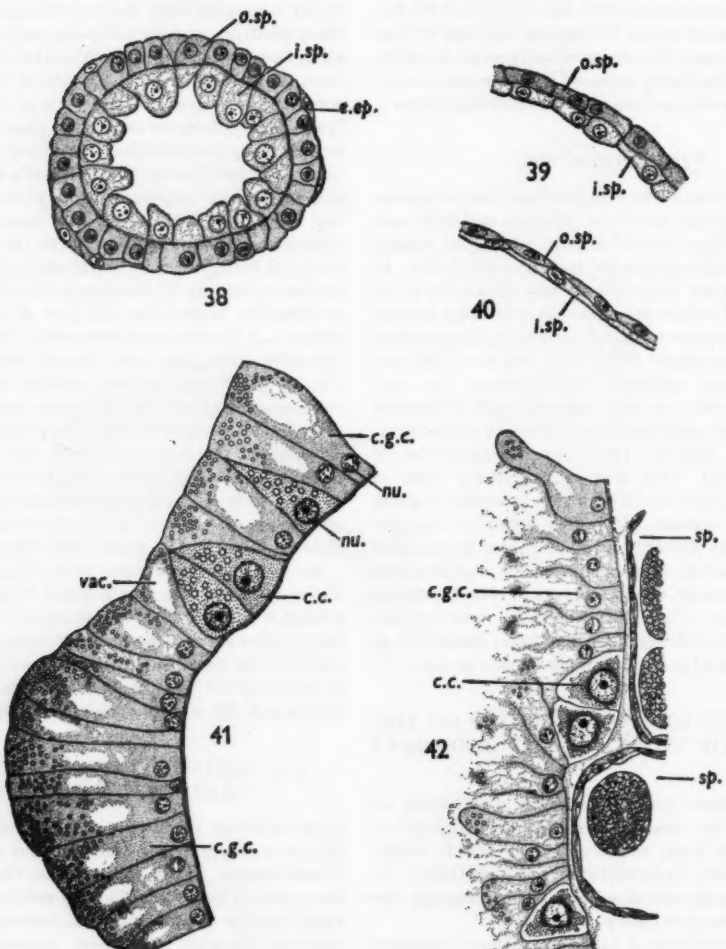
The digestive gland of *Lymnaea pereger* consists of groups of blindly ending hepatic tubules separated from one another by interstitial tissue. The walls of the tubules consist of cells of two types, the columnar glandular cells and the so-called calcareous cells (Fig. 41). The columnar cells are by far the more numerous and form a single layer of cells bounded on the outside by the limiting layer of the tubule. Each possesses a nucleus situated in the proximal part of the cell. The cytoplasm is densest at the distal extremity, and secretory vacuoles occur near the central region of the cells. The calcareous cells are less numerous; they occur singly or in groups of two or three and are situated proximally between the columnar cells, rarely reaching the lumen of the tubule. The cytoplasm in these cells is dense and the nucleus large.

The developing sporocysts lie between the tubules where they gradually destroy the interstitial tissue. They multiply rapidly and eventually occupy almost all the intertubular spaces. When the sporocysts have been in contact with the walls of the tubules for some time changes are apparent in the cells. Cytolysis of the columnar cells takes place at their distal extremities, the cytoplasm escaping into the lumen of the tubules. The nucleus, normally at the proximal end of the cell, migrates slightly towards its distal end (Fig. 42). This latter has been explained by Agersborg (1924) as being due to the fact that food material brought to the hepatic tubules by the blood stream is used up by the parasite so that the nucleus is forced to migrate to the part of the hepatic cell where the ferments are secreted. Vacuolization of the cells takes place and the cell walls degenerate. Finally there may be local or total histolysis of the tubular epithelium. At the same time changes take place in the calcareous cells. They do not break down as early as the columnar cells, but the cytoplasm shrinks away from the wall and collects around the central nucleus. It is possible that toxic substances are given off by the sporocyst which diffuse into these cells, bringing about the altered conditions.

In extreme cases very little functional hepatic tissue remains, the whole gland being replaced by a mass of sporocysts and escaping cercariae. Whether the tubules are capable of regeneration if the mollusc survives the period of infection is not known, though it is so in the case of *L. truncatula* infected with rediae and cercariae of *Fasciola hepatica* (Rees, 1931).

IX. SECOND INTERMEDIATE HOST AND CYST FORMATION

In an attempt to find the second intermediate host infection experiments were carried out with those invertebrates which occurred in the pools in the farmyard. These were *Tubifex tubifex* (Müller), and three species of insect larvae, namely, *Chironomus riparius* Meigen, *Culicoides stigma* Meigen, and *C. nubeculosus* Meigen. The writer is indebted to Mr Paul Freeman of the Natural History Museum for identifying these insects and one other species, *Anatopynia* (*Psectrotanypus*) *varius* Fabr., obtained from another locality and used when the supply from the farm ceased owing to drying of the pools. Adult stages were bred out in the laboratory and sent to Mr Freeman for identification. As a preliminary, five larvae of *Chironomus riparius* were placed in a watch-glass with a large number of cercariae which had recently emerged from infected snails. There was a rapid penetration by large numbers of cercariae into the chironomid larvae causing



Figs. 38–42.

Fig. 38. Transverse section through wall of young sporocyst.

Fig. 39. Transverse section through wall of older sporocyst.

Fig. 40. Transverse section through wall of sporocyst distended with cercariae.

Fig. 41. Part of wall of tubule of digestive gland of *Lymnaea pereger* showing normal structure.

Fig. 42. Part of wall of tubule of digestive gland of *Lymnaea pereger* showing effects of parasitization.

their death in periods varying from 1½ to 2½ hr. Up to 120 cercariae were counted in these dead chironomids. Similarly, larvae of the two species of Culicoides were exposed to the same conditions and again large numbers of cercariae entered them, causing death in from 2 to 3½ hr., up to 52 cercariae being counted in each insect larva. Specimens of *Tubifex tubifex* were treated similarly, but no cercariae penetrated into them. They crawled over the surface of the worms but made no attempt to enter, leaving them after a short time to resume active swimming in the surrounding water.

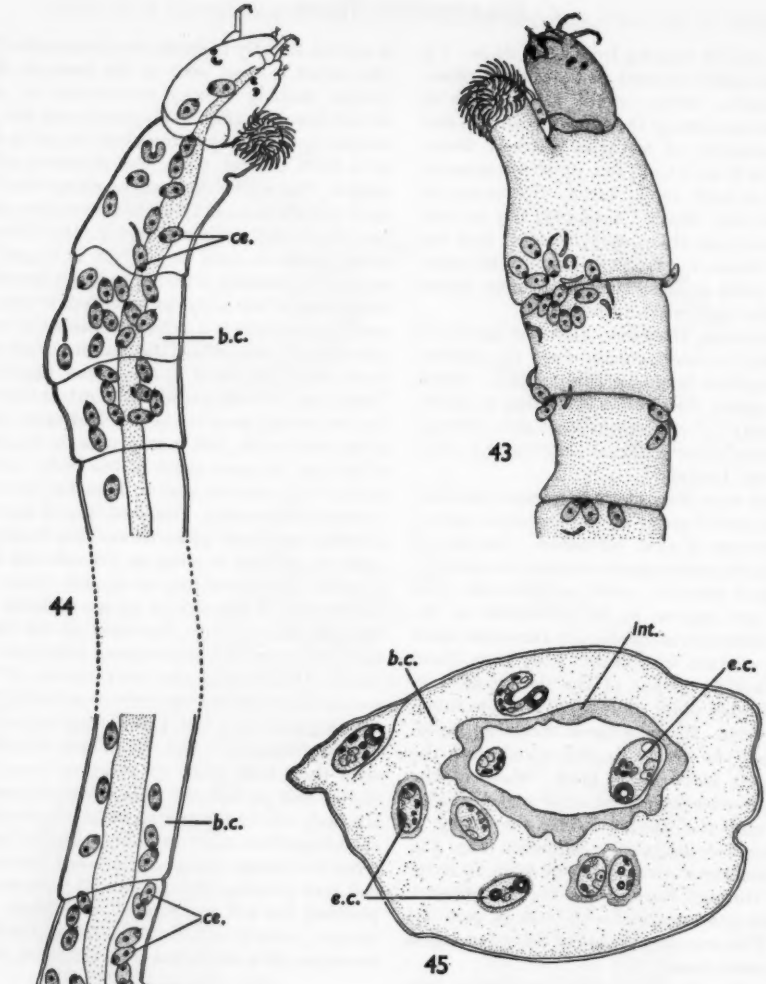
It seemed evident, therefore, that the larvae of chironomids and ceratopogonids were the normal second intermediate hosts of this species. Other species of the genus *Plagiorchis* encysting in chironomid larvae are *P.* (*P.*) *proximus* McMullen (1937*a*), *P.* (*P.*) *maculosus* Nöller & Ullrich (1927) and *P.* (*M.*) *muris* McMullen (1937*a*).

Observations were then made in greater detail to examine the mode of entry of the cercariae and to follow the process of cyst formation. Larvae of *Chironomus riparius* were again exposed to numbers of cercariae and watched under a binocular. The cercariae do not appear to be attracted to the chironomids, but swim at random in the water until they come in contact with them. They then crawl over the surface by means of the two suckers in search of a suitable point of entry. The time taken to find this varies. In some cases it is very rapid, while in others the cercariae will crawl over the insect larva for some little time. Most of the cercariae accumulate at the anterior and posterior extremities, and the point chosen for entry is always between two successive segments (Fig. 43). In one instance three cercariae were seen to enter very rapidly through the anus of the chironomid, boring through the intestinal wall later to gain the body cavity. This was only observed on one occasion and does not seem usual.

To examine the actual process of penetration, one larva of *C. riparius* was placed on a slide under a cover-slip with twenty cercariae and watched under a microscope using a × 10 ocular and ⅔ in. and ⅙ in. objectives. The movements of the chironomid were restricted by the cover-slip and the whole sequence of events could be followed. Having found a suitable spot for entry, the cercaria attaches itself firmly by both suckers placed fairly close to one another. The ventral sucker does not change its position during the process, but the oral sucker may move slightly backwards and forwards as the slit is being made in the cuticle of the host. The slit is made by the stylet, a remarkably efficient organ for the purpose. The stylet, which is normally placed horizontally, is turned downwards towards the mouth, presumably by the action of muscles attached to it. As already noted, the tip of the stylet is curved slightly towards the dorsal side (Fig. 21*b*). The stylet is then used in the manner of a small scalpel making cutting movements in a ventro-dorsal direction (Fig. 21*c*), puncturing the cuticle at several points along a line about equal in length to, or a little greater than, the diameter of the oral sucker. The stylet then works along this line again until the slit is complete, the oral sucker shifting its position slightly if necessary. Entry of the cercaria takes place as soon as the slit is completed. The anterior extremity is thrust through the slit and the remainder of the body drawn through without pause until the cercaria is completely inside the body of the chironomid, after which the margins of the slit, which have been stretched apart, come together again. Entry may be very rapid, the shortest time observed for the completion of the slit and entry of the cercaria was 2 min., but it may take up to a maximum of 13 min. in some parts of the body. Penetration seems to be easiest and to take less time near the posterior extremity. The shedding of the tail of the cercaria may take place at varying times. In some cases it is shed as soon as the cercaria begins the process of penetration, or at any time up to the completion of the slit, or as the cercaria is passing through the slit into the body of the chironomid. In a few cases the tail remains attached until after entry. Once inside the body cavity of the chironomid the cercariae crawl about actively for a period varying from ½ to 1 hr. before they begin the process of cyst formation (Fig. 44). Those which have not shed their tails prior to entry do so at any time during this period, the tails remaining active inside the body cavity for as long as 24 hr. after entry. The shedding of the tail is brought about by the secretion from the dorsal glands in the neighbourhood of the tail root passing into the caudal pockets, gradually pushing the tail away until it is finally shed. The caudal pockets can be seen to be filled with the secretion after the tail is shed (Figs. 26, 27).

X. REACTIONS OF CHIRONOMIDS TO PENETRATION BY CERCARIAE

No reaction was observed while the cercariae were crawling over the surface of the chironomid larvae, but once the process of penetration began there is a marked response. The chironomids undergo violent spasmodic movements with constant ventral flexions of the posterior end. The surface of the body is explored by the mouth and 'brushed' by the thoracic appendages, the spines of which may be effective in removing the cercariae temporarily, though they may return after a short swimming period in the water in the neighbourhood of the host. Some cercariae are actually picked off by the mouthparts and swallowed. Others, which are swimming in the water, may also be swallowed and are often found in



Figs. 43–46.

Fig. 43. Anterior extremity of chironomid larva showing accumulation of cercariae on the surface in the junctions between the segments.

Fig. 44. Anterior and posterior extremities of chironomid larva showing numerous cercariae in body cavity, not yet encysted.

Fig. 45. Transverse section through chironomid larva showing encysting cercariae in body cavity and alimentary canal.

Fig. 46. Three cercariae in process of encysting, from body cavity of chironomid larva.

the alimentary canal in sectioned specimens (Fig. 45). Some of these are able to penetrate the gut wall and reach the body cavity, while others begin the process of cyst formation. If these cysts are fully formed in the gut and if the chironomid survives, they will probably be passed out with the faeces in course of time. The chironomid is not able to explore all parts of the body and all the cercariae cannot be picked off. The 'cleaning process' is disturbed by pricking sensations elsewhere and so is alternated with violent spasmodic flexions. Many cercariae therefore are able to enter in a short time before the host can deal with them. Cercariae do not seem to be dislodged by the flexions of the body once the suckers have gained a firm hold. After the cercariae have entered, the behaviour of the chironomids returns to normal provided the number entering is not excessive. In the latter case the movements gradually slow down until death takes place. Such larvae gradually lose their red colour and at the time of death are quite colourless; presumably they 'bleed to death' through the numerous slits made in the body wall.

Cyst formation

When very large numbers of cercariae penetrate, bringing about the death of the chironomid larva very rapidly, the process of encystment is not completed. It is not known how many simultaneous penetrations the insect larvae can survive, but up to twenty do not kill them within a period of 1 week which was the longest time infested specimens were kept.

The process of encystment was examined in living specimens and in sections of infested chironomids. For this latter purpose the chironomids were killed in hot Carnoy's fluid. Sections were cut at 5 μ and stained with Mallory's triple stain.

When the cyst is about to be formed, the body of the cercaria curls up with the concavity ventral and the mouth in contact with the excretory pore, forming an almost spherical body. It then begins to revolve head forwards and fairly rapidly in the one direction, and at the same time a secretion is poured out from the dorsal surface, forming a delicate membrane around the body. This secretion seems to be derived from the dorsal gland cells, the staining reaction being similar. At intervals, the whole body changes its position through an angle of 90° and continues to revolve so that the spherical shape of the cyst may be maintained while it is hardening and the inside can be made smooth. This process continues for about an hour, the structure of the body of the cercaria remaining unaltered and the stylet still in position. When very large numbers of cercariae are present, they may be so close together that the secretion of the dorsal glands becomes contiguous and several cercariae are embedded in a mass of this secretion (Fig. 46). In such cases the process of encystment may cease at this point, the cyst never being completely formed, but in some instances the beginning of the formation of the cyst wall proper is seen. In normal cases after the formation of the initial membrane the movements of the cercaria slow down and become irregular. A secretion is now exuded from the cystogenous gland cells and accumulates as a thin layer lining the original membrane. The cercaria twists around inside the developing cyst, presumably distributing the cystogenous material in a uniform layer. The complete formation of the cyst requires a period of about 12 hr. By this time a mass of excretory granules has collected in the excretory vesicle which is distended and appears in living specimens as a trilobed dark mass. The stylet is shed and can be seen lying inside the cyst. The cyst with its contained metacercaria is now assumed to have reached the infective stage (Fig. 28). Slow movements of the metacercaria inside the cyst continue up to a week after cyst formation and probably longer, but this was the longest time that they were kept under observation. The cyst is spherical in shape and measures 0·103 mm. in diameter. If the cyst is in such a situation that it is compressed by the organs of the host, e.g. when it is at the extreme anterior end, it may be oval in outline, measuring 0·10 × 0·12 mm.

The process of penetration and of cyst formation is essentially the same in the other insect larvae studied, namely, *Culicoides stigma*, *C. nubeculosus* and *Anatopynia* (*Psectrotanypus*) *varius*.

Some infested specimens of *Chironomus riparius* were kept to ascertain if they would complete their metamorphosis. One pupated but died in the pupal stage. It contained fifteen cysts. Four completed their metamorphosis and contained in the imaginal stage one, two, three and nine cysts respectively. In one instance a single cyst was found free in a discarded pupal case. Whether the cysts are still infective in the imago is not known, as no feeding experiments were made with them.

XI. DEFINITIVE HOST, EXPERIMENTAL INFESTATION OF TURKEY POULTS

As already stated, it is almost certain that the turkey is not the natural host of *Plagiorchis* (*M.*) *megalorchis*, but as the parasite was found to infest turkeys it was decided to use turkey poults for experimental infestation. Six turkey poults, hatched out on 3 July 1950, were obtained for the purpose. These were procured by Mr L. E. Hughes and Mr F. Jordan and housed by them in the veterinary laboratory. The actual feeding of the experimental birds with the insect larvae was done by them in the presence of the writer, who is most grateful for their assistance, as without it the experimental section of

the work could not have been done. The post-mortem examinations were made by Mr F. Jordan and the writer.

On 7 July turkey 1 was given 50 infested larvae of the two species of Culicoides and three infested larvae of *Chironomus riparius*. These larvae had been exposed to large numbers of cercariae 48 hr. previously and most had died due to heavy penetration. This bird died on 11 July from some unknown cause. No trematodes were found in the gut. Turkey 2 was given 50 infested larvae of the two species of Culicoides on each of three successive days, namely, 7, 8 and 9 July. These, too, were for the most part dead specimens. The bird was killed on 12 July, but no trematodes were found.

The lack of success in these two cases was probably due to the fact that the cysts do not form properly if the insect larva is killed by a mass penetration by cercariae. It was decided, therefore, to restrict the number of cercariae entering the insect larvae and to feed the latter alive to the turkey poults at varying times after the completion of cyst formation. Turkey 3 was given 70 live insect larvae comprising *Chironomus riparius* and *Anatopynia* (*Psectrotanypus*) *varius* each containing about 10 encysted cercariae on 20 July. These larvae had been exposed to infection on 14 July and the contained cysts were therefore 6 days old. On 24 July the same bird was given 90 more live larvae, each containing again about 10 encysted cercariae. These were divided into three groups of 30 each, each group having been exposed to cercariae on 19, 20 and 21 July respectively. The contained cysts were therefore from 3 to 5 days old. This bird had therefore been given a number of encysted cercariae varying in age from 3 to 6 days. The bird was killed on 29 July and 5 sexually mature specimens of *Plagiorchis* (*M.*) *megalorchis* were recovered from the small intestine.

Turkey 4 was given 40 live insect larvae comprising *Chironomus riparius* and *Anatopynia* (*Psectrotanypus*) *varius*, each containing about 10 cysts on 21 July. Some of these cysts were 3 days old and some 4. A post-mortem examination on 30 July revealed 4 mature trematodes in the small intestine.

In both these turkey poults the adult worms were found in the same region of the small intestine, namely, from 14 to 24 in. behind the gizzard.

Two control birds were kept. There were no trematodes in one of these examined.

The adult trematode recovered was identified as the same species as that originally obtained from the turkey poults which had died on the farm. The infestation, however, in the experimental birds was not heavy enough to reproduce symptoms of disease. Probably the resistance of the birds was considerably greater as they were being well cared for and their diet scientifically planned.

XII. SUMMARY

1. Large numbers of *Plagiorchis* (*M.*) *megalorchis* n.nom. were found in the intestine of four turkey poults of a total of thirteen which had died on a farm in Radnorshire. Presumably the presence of the parasites was the cause of death.

2. The anatomy of the adult worm is described.

3. The first intermediate host of the parasite is *Lymnaea pereger*, and the second intermediate host any of the following species of insect larvae: *Chironomus riparius* Meigen, *Culicoides stigma* Meigen, *Culicoides nubeculosus* Meigen and *Anatopynia* (*Psectrotanypus*) *varius* Fabr.

4. The larval stages of the worm are described.

5. The life cycle has been demonstrated experimentally by the feeding of insect larvae containing encysted cercariae to turkey poults.

6. The turkey is probably not the normal host of *Plagiorchis* (*M.*) *megalorchis*. It is most likely that it occurs naturally in some species of wild bird, but this is not yet known.

XIII. KEY TO LETTERING OF FIGURES

(All drawings are semi-diagrammatic.)

a.n.	anterior nerves
b.c.	body cavity of chironomid larva
br.	brain
c.	cirrus
c.c.	calcareous cell of hepatic tubule
c.ch.	central chamber of Mehlis's gland
c.e.d.	caudal excretory duct
c.g.c.	columnar glandular cell of hepatic tubule
c.p.	caudal pocket
c.s.	cirrus-sac
ce.	cercaria in body cavity of chironomid larva
cy.gl.	cystogenous gland cell
cy.w.	cyst wall
d.gl.	dorsal gland cell
d.n.	dorsal nerve
d.v.m.	dorso-ventral muscles
e.	egg
e.c.	encysting cercaria
e.ep.	external epithelium of sporocyst wall
ex.p.	excretory pore
ex.ves.	excretory vesicle
g.r.	genital rudiment
i.sp.	inner layer of sporocyst wall
int.	intestine
l.c.	Laurer's canal
l.n.	lateral nerve
m.gl.	Mehlis's gland
m.o.p.	muscles from oral sucker to pharynx
m.v.d.	median yolk duct
mt.	metraterm
mt.gl.	gland cells around metraterm
nu.	nucleus
o.s.	oral sucker
o.sp.	outer layer of sporocyst wall

oe.	oesophagus
ov.	ovary
ovd.	oviduct
ph.	pharynx
pph.	prepharynx
pro.	prostate gland cells
s.cy.gl.	secretion from cystogenous glands
s.d.c.	secretion from dorsal glands in caudal pocket
s.d.gl.	secretion from dorsal glands
sp.	sporocyst
sp.ut.	sperms in descending uterus
st.	stylet
st.gl.	stylet gland cells
t.	tail
t.1	anterior testis
t.2	posterior testis
ut.a.	ascending limb of uterus
ut.d.	descending limb of uterus
v.ef.	vas efferens
v.gl.	ventral gland cell
v.n.	ventral nerve
v.s.	ventral sucker
vac.	vacuole.
ves.sem.	vesicula seminalis
vit.	yolk glands
vit.d.	yolk duct

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SPIRAL TORSION IN *TAENIA TAENIAEFORMIS* (BATSCH, 1786) CAUSED BY THE KNOTTING TOGETHER OF TWO WORMS

By GWENDOLEN REES, D.Sc.
Department of Zoology, University College of Wales, Aberystwyth

(With 24 Figures in the Text)

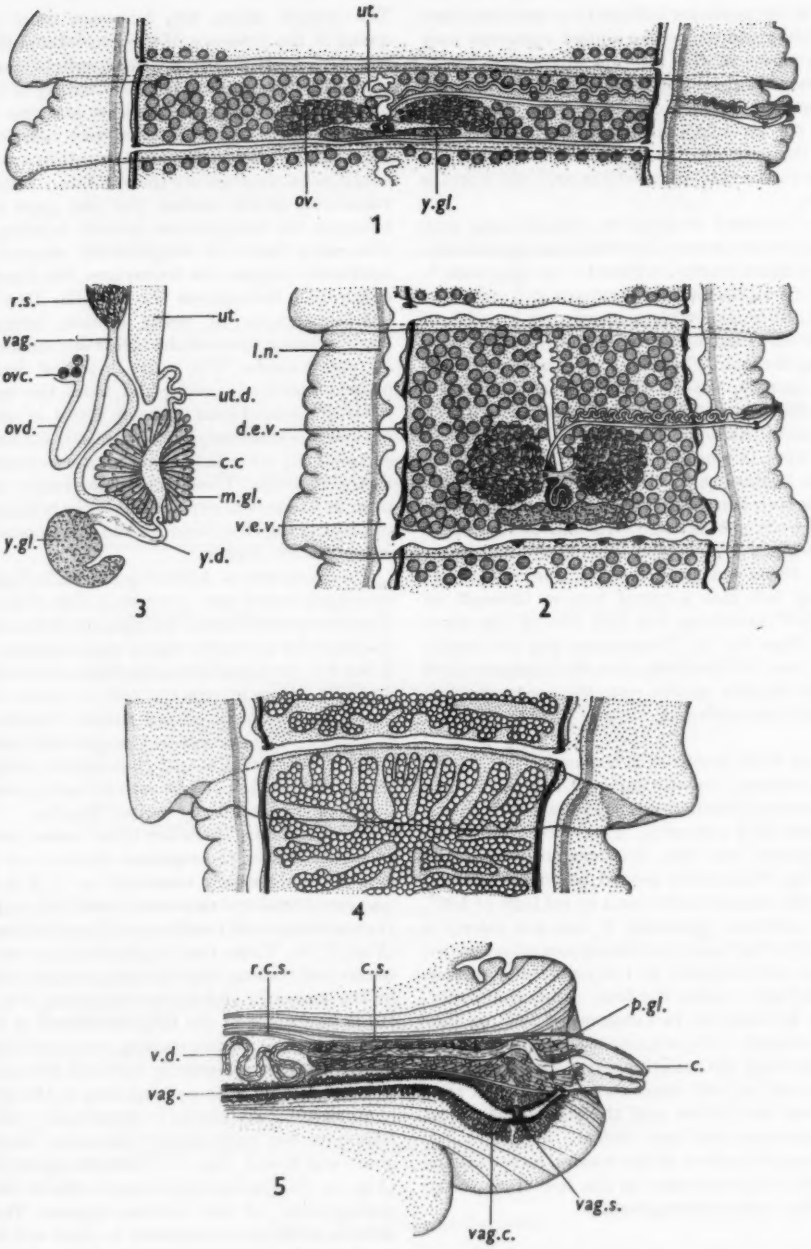
The larval stage of *Taenia taeniaeformis*, namely, *Cysticercus taeniae-taeniaeformis*, has recently been described by the writer (1951), a detailed account being given of the musculature and of the excretory and nervous systems of the scolex and strobila. In addition, some abnormalities in structure were noted, chief of which was the spiral torsion observed in some specimens and which involved the musculature, excretory and nervous systems of the strobila but not the surface layers. A search was then made for some adult specimens in order to find whether the same abnormality could be seen and to ascertain what the effect of such a torsion would be on the genitalia in the mature and gravid regions. Several adult specimens, from the intestine of a cat, were given to the writer by Mr L. E. Hughes, veterinary investigation officer. Unfortunately, none of these specimens showed the identical spiral torsion found in the larva, but two of them had become knotted together, presumably at an early stage, and were considerably distorted in the regions involved in the knot. This distortion was again of a spiral nature but involved the entire proglottid and not only the internal structures as was found in the type of spiral torsion common in the larval stage of this species. The condition was not merely a turning over of the strobila but an abnormal spiral growth brought about by the continued constriction on the body where the two worms were twisted around one another. So here again, in the adult stage, spiral torsion is evident but from a different cause and involving the whole of the relevant segments from the surface layers to all the contained organs.

Five specimens of *Taenia taeniaeformis* were examined. Three of these were separate from one another and of normal appearance, the remaining two were knotted together at about half way along the strobila in each case. The knot was a complicated one and was pulled quite tightly. It must have been formed at a fairly early stage as the normal growth of the strobilae had obviously been considerably impeded in the regions involved. It is extremely unlikely that the knot could ever have been unravelled in the gut of the host, but it would of course have separated off from the strobila eventually as this region became gravid and the proglottides broke away.

On removal from the host the knot was unravelled carefully without breaking the strobilae. All the worms were then fixed in 5% formol and 'normal' and 'abnormal' strobilae examined. Whole mounts were stained with Mayer's paracarmine and serial sections cut at 5 μ were stained with Heidenhain's azan.

The three 'normal' worms measured 110·0, 70·0 and 57·0 mm. in length, with a maximum diameter in each case of 7·0 mm. The number of segments constituting the strobila was 371, 187 and 197 respectively. The measurements of the scolex and its constituent parts agree with those already given for the cysticercus as also do the number and arrangement of the hooks.

Even in the so-called normal strobilae there was some variation in shape of the individual proglottides. Normally, the segments are all broader than they are long (Fig. 1), but in the first specimen there was a sudden change in the shape of the segments near the posterior end of the strobila. A series of segments comprising the last eight were almost square in outline (Fig. 2), the first of them measuring 3·44 mm. long and 3·0 mm. broad, about half the maximum breadth attained in the earlier part of the strobila. The change in shape is a sudden one, but the three segments immediately in front of the series show a slight lengthening and narrowing. The first three segments of the series were mature and the last five gravid. In the second specimen a similar variation in the shape of the segments was again evident. In this case there were three isolated regions along the length of the strobila where the segments suddenly become longer and narrower than usual. The last eleven segments were almost square in outline, measuring on an average 1·88 mm. long and 2·70 mm. wide in contrast to those segments immediately in front which measured 0·6 mm. long and 5·5 mm. wide. There were no gravid segments in this strobila. In the third specimen there was again the same variation. No such irregularity in the shape of the proglottides was observed in the larval specimens examined previously, the segmentation there being remarkably even. The sudden changes in the shape of the segments in the three adult worms examined is presumably due to a sudden alteration in the growth rate. It is not merely a lengthening of the



Figs. 1–5. *Taenia taeniaeformis.*

Fig. 1. Mature segment, normal shape, dorsal view.
Fig. 2. Mature segment, square in shape, ventral view.
Fig. 3. Reconstruction of female genitalia, side view.
Fig. 4. Anterior extremity of gravid segment.
Fig. 5. Cirrus sac and vagina.

segments at the posterior extremity to accommodate the expanding uterus, as the square segments may occur anywhere in the strobila interspersed with those of more normal appearance. They inevitably become carried backwards during the growth in length of the strobila and eventually will become terminal. What causes this apparent change or series of changes in the rate of growth in any one worm is not known.

The two knotted worms, on straightening out, were found to have a curiously distorted appearance. The first of these worms, referred to as specimen 1, consisted of a scolex and 242 segments and measured 130·0 mm. in length with a maximum breadth of 7·0 mm. Four constricted regions were revealed on unravelling the knot. The first of these involved three segments, namely, 189–190 (Fig. 10), the second two segments, 194 and 195, and the third and fourth one segment each, namely, 200 and 203. All these segments were in the late mature stage where the uterus was beginning to fill with eggs. In the last three of the four constricted regions the proglottides concerned were merely narrowed and elongated to varying degrees but in the first, involving three segments, there was not only a narrowing and lengthening but also a spiral torsion through an angle of 180° involving the first two of the three segments (Figs. 10, 11). This torsion was not merely a turning over of the body, but the segments had been forced to grow in this way, the twist affecting permanently the surface layers and all the contained organs.

The other worm involved in the knot, specimen 2, was much shorter, measuring 56·0 mm. in length with a maximum breadth of 6·0 mm. and comprising a scolex and 173 segments. There were two constricted regions, the first, involving segments 158 and 159 (Fig. 19), and the second, segments 162 and 163. In both regions there was a spiral turn of 180°. As in the previous specimen, it was not merely a turning over of the body but the segments had grown in this distorted manner as the result of pressure. In this specimen, unlike the first, there was a complete turn, between the two regions, of 360°, so that the terminal part of the strobila was restored to the same position as the anterior part. In specimen 1 there was only a half turn, through 180°, so that viewed from the surface half the strobila is dorsal surface uppermost and half ventral.

To ascertain the effect of this torsion on the organs in the body, it is necessary in the first instance to examine the normal arrangement.

NORMAL STROBILA

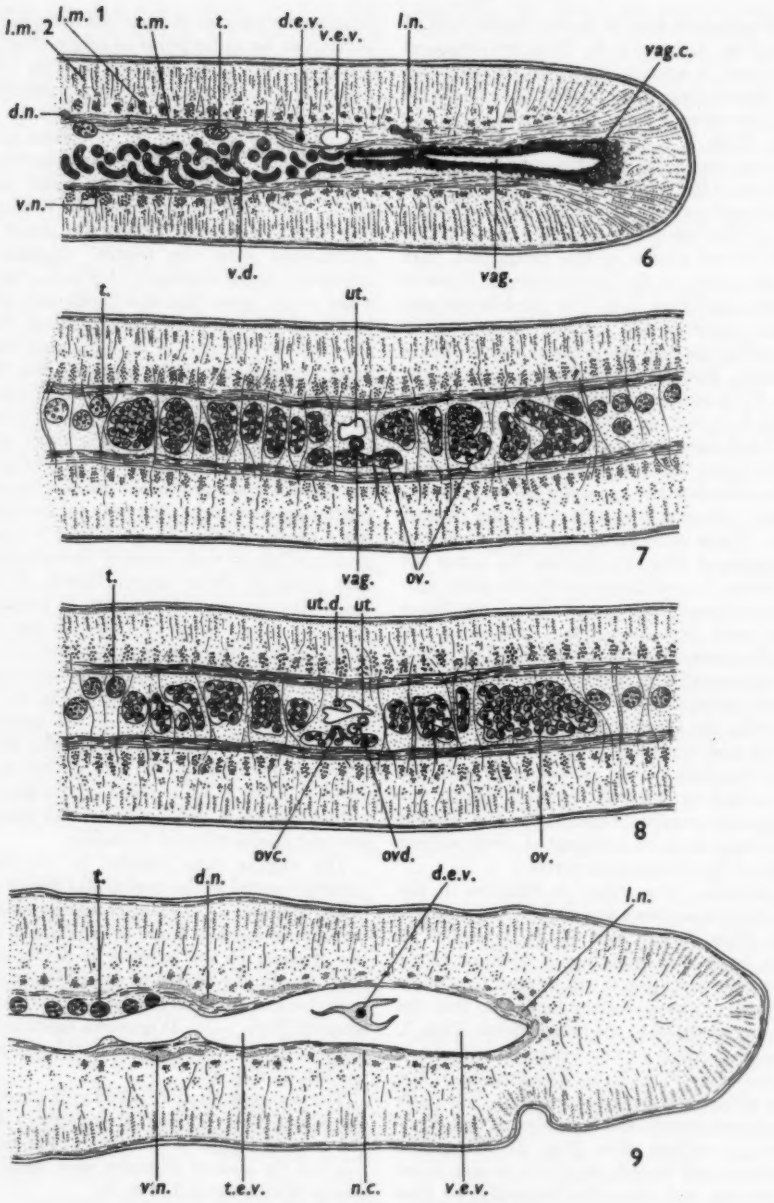
Musculature. A transverse section through the mature region shows that the musculature is essentially the same as in the body of the larva. The muscle layers are, however, more restricted owing to the presence of the genitalia in the medullary region. In addition to a superficial musculature there is a deep musculature consisting of two layers of longitudinal muscle bundles and one of transverse (Figs. 6–8). The transverse muscles form a dorsal and ventral plate in each segment and are not continuous throughout the strobila. Laterally the transverse fibres spread out and pass in groups between the longitudinal muscle bundles (Fig. 6). The inner layer of longitudinal muscles lies immediately outside the transverse, the bundles being continuous throughout the strobila. This layer has the appearance of being double, larger muscle bundles being towards the inside and smaller bundles on the outside. The outer layer of longitudinal muscle bundles is separated from the inner by a layer of parenchyma and the fibres of which they are composed are smaller (Figs. 6–9). Radial muscles occur in the cortical region and dorso-ventral fibres in the medulla. These latter are better developed than in the larva, but are unevenly arranged owing to their being displaced by the development of the reproductive organs.

Excretory system. A small dorsal and a large ventral excretory vessel pass down each side of the strobila. The two ventral vessels are connected by a transverse vessel at the posterior end of each segment (Figs. 1, 2 and 6). As in the larva, the transverse vessel forks laterally before it joins the ventral vessel, the dorsal vessel passing through the ring so formed (Figs. 1, 2, 4 and 9). The transverse vessel is very much larger than in the larval stage; it is slightly lobed and in transverse section can be seen to occupy most of the depth of the medullary region (Fig. 9).

Nerve system. There are three lateral nerve cords on either side of the segment situated on the outer side of the excretory vessels (Figs. 1, 2, 4, 6 and 9), and two dorsal and two ventral nerves lying between the transverse and inner layer of longitudinal muscles (Figs. 6, 9). These ten longitudinal nerves are connected with one another by a ring-shaped commissure at the posterior end of each segment (Fig. 9). This commissure lies in the neighbourhood of the transverse excretory vessel, passing outside the transverse muscles which are greatly reduced here as they are not continuous from one segment to the next.

Genitalia. As already mentioned, the mature segments can vary greatly in shape. They may be short and broad (Fig. 1) or almost square in outline (Fig. 2). This difference in shape affects the size and arrangement of the internal organs. The genital atrium alternates irregularly to right and left of the strobila. It lies just in front of the middle of the lateral margin of the segment, its relative position being constant even when the segments are of different lengths (Figs. 1, 2).

Male genitalia. There are numerous testes which



Figs. 7–9. *Taenia taeniaeformis.*

Fig. 6. Half transverse section through mature segment showing vas deferens and vagina passing ventrally to lateral excretory vessels and nerves.

Fig. 7. Transverse section through central region of mature segment showing uterus, vagina and ovary.

Fig. 8. Transverse section through central region of mature segment showing ovicapt, oviduct, uterus and uterine duct.

Fig. 9. Half transverse section through posterior end of mature segment showing lateral and transverse excretory vessels and nerve commissure.

are evenly distributed and lie in the dorsal half of the medulla (Figs. 1, 2 and 6–9). They are arranged for the most part in a single layer but occasionally may be two layers deep. They extend backwards on either side of the ovary where they are limited to the lateral fields (Figs. 1, 2, 7 and 8), but behind the yolk gland they may form a continuous row again, being restricted to the central region of the segment in the neighbourhood of the transverse excretory vessel (Fig. 9). The vas deferens is very much coiled and runs from the centre of the proglottid, just anterior to the ovary, to the lateral margin where it enters the cirrus sac (Figs. 1, 2). The vas deferens and cirrus sac are anterior and slightly dorsal to the vagina, the openings into the genital atrium being similarly placed. Both vas deferens and vagina pass ventrally to the lateral excretory vessels and lateral nerves (Figs. 1, 2 and 6). The cirrus sac is short, measuring 0·447 mm. in length with a maximum breadth of 0·10 mm. There is no external or internal vesicula seminalis, but the vas deferens on entering the cirrus sac is thrown into coils which are packed with sperms. These coils straighten out when the cirrus is evaginated (Fig. 5). Within the cirrus sac the vas deferens is continuous with the pars prostatica, ductus ejaculatorius and cirrus, the latter being armed with delicate spines. Prostate gland cells fill up the interior of the cirrus sac. The cirrus sac, in spite of its small size, is provided with retractor muscle fibres inserted into the proximal end and continuous with the transverse muscles of the segment dorsally and ventrally. In some cases, when the cirrus is evaginated, the lateral margin of the proglottid around the genital atrium is drawn out into a prominent papilla (Figs. 1, 5); the genital atrium itself may even be evaginated, thus adding to the length of the evaginated cirrus.

Female genitalia. The ovary is situated in the posterior half of the segment and slightly towards the ventral side (Figs. 1, 2, 7 and 8). It is bilobed in surface view, each lobe being again lobed or sacciform. In transverse section it can be seen to occupy the whole depth of the medullary region, so that the testes are here restricted to the lateral fields (Figs. 7, 8). The median portion of the ovary or isthmus is ventral in position (Figs. 7, 8). The outline of the ovary varies with the shape of the segment. When the segment is almost square, the two lobes of the ovary are rounded in surface view (Fig. 2). When the segment is short and broad, each lobe is also short antero-posteriorly and expanded laterally, so that the resulting shape of each lobe is transversely oval or club-shaped (Fig. 1). In both cases it does not extend forwards beyond the main reproductive ducts. The oviduct arises as an ovicapt from the postero-dorsal side of the isthmus (Figs. 3, 8). It runs backwards in the middle line for a short distance, then turns forwards again to form an ascending limb running dorsally to, and parallel with, the descending portion as far as its point of origin (Fig. 3). Here it receives the vagina anteriorly. The oviduct then turns backwards again and runs dorsally to the first loop as far as the anterior margin of the yolk gland, the oviduct describing in its course so far an N-shaped bend. It then runs dorsally across the medullary region between the yolk gland and Mehlis's gland, receiving the yolk duct as it turns forwards into Mehlis's gland. Within the gland it becomes continuous with the central chamber emerging anteriorly as the uterine duct which, after describing a few coils, opens into the dorsal side of the uterus just in front of its posterior extremity. The uterus in the mature segment is an elongated tube running antero-posteriorly in the centre of the dorsal half of the medulla (Figs. 1, 2, 7 and 8). If the segment is square in outline the uterus lies in a straight line (Fig. 2), but if the segment is short the uterus is thrown into folds from side to side (Fig. 1). As the segments become gravid the uterus gives off branches beginning anteriorly. The fully formed gravid uterus has a fairly constant shape. There is a median stem giving off about eight pairs of lateral branches which may again divide or become lobed. At the anterior end of the segment the pattern of branches is fairly constant, there being two median and two antero-lateral stems, which eventually divide to give a row of about twelve blindly ending branches all terminating along the anterior margin (Fig. 4). The uterus occupies the whole depth of the medulla, and in transverse section the branches can be seen to be only one layer deep. In the gravid region of the strobila there is an increase in the size of the individual segments distinct from the irregularities in size and shape referred to earlier.

The vagina runs parallel to the vas deferens, passing ventral to the uterus and dorsal to the ovary (Figs. 1, 2 and 7). It is surrounded along its length by a dense mass of small cells (Figs. 5, 6), and near the opening to the exterior is a vaginal sphincter (Fig. 5). In the centre of the proglottid the vagina turns backwards and enlarges to form a receptaculum seminis (Figs. 1–3). It narrows again before it opens into the oviduct at the apex of the ascending limb of the latter (Fig. 3).

The compact slightly lobed yolk gland lies behind the ovary. The yolk duct arises antero-dorsally from the central region of the gland, runs parallel to the oviduct for a short distance, and opens into it as it enters Mehlis's gland (Fig. 3).

ABNORMAL STROBILA

As already mentioned, two of the worms, specimens 1 and 2, were knotted together resulting in an abnormal growth in the parts of the strobilae involved in the knot.

Specimen 1. In this worm there are four constricted regions in the strobila, the first, involving three segments, showing a spiral torsion involving the surface layers and all the contained organs (Figs. 10–18). The twist occurs in the latter half of the first and early part of the second segments (Figs. 10, 11). The first segment narrows abruptly at about one-third of its length, the second is narrow anteriorly, broadening slightly posteriorly, and the third, narrow at first, broadens rapidly posteriorly and is followed by segments of normal appearance. All three of these segments are considerably longer than usual. The surface of the cuticle is deeply furrowed, the furrows in the posterior half of segment 1 and anterior half of segment 2 running in a spiral direction around the body (Fig. 10). Segment 1 is seen in ventral view and segments 2 and 3 in dorsal view. This twisting is not merely a turning over of the strobila, but has resulted from a distorted growth. Most of the distortion is apparent in the first segment, so this segment only will be considered.

The genital atrium normally on the lateral margin of the proglottid is in the middle of the dorsal surface (Figs. 11, 12). It is situated on an extension of the cortical region which grows towards the centre from the lateral margin, taking the reproductive ducts with it (Fig. 12). A similar fold occurs along the ventral side of the segment. A series of transverse sections through this segment shows the effect of the torsion on the general shape. The deep furrowing of the surface is apparent in all sections, being most marked at the posterior end (Figs. 12–18). At the anterior end the sections have a fairly normal outline except for the dorsal and ventral prolongations of the cortex. The musculature excretory and nervous systems are as usual. Proceeding backwards, the left lateral margin grows over dorsally towards the right side, so that the ventral surface becomes convex and increased in area and the dorsal surface concave and decreased in area (Figs. 13–16). The musculature is affected by the deep furrowing, so that the band of longitudinal muscles becomes indented opposite the furrows and bulges out between them. Towards the posterior end of the segment the torsion is completed fairly rapidly (Figs. 15–18). In the region of the yolk gland (Fig. 15) the ventral surface is extensive and forms by far the greater part of the circumference of the section, the dorsal surface being reduced to a deep concavity with folds and ridges. The longitudinal muscles are increased in extent ventrally and reduced dorsally. The lateral nerves and excretory vessels of the left side now occupy a position equivalent to what was originally mid-dorsal. Behind the yolk gland this is more pronounced, the lateral excretory vessels and nerves of both sides now lying directly above one another in a dorso-ventral direction (Fig. 16). This is the point at which the actual torsion occurs. The ventral surface now occupies nearly the whole circumference of the section, the dorsal surface being reduced to a deep concavity on the right side. More posteriorly still, the turn is completed. The convex ventral surface now lies dorsally, and the deeply furrowed dorsal surface ventrally (Fig. 17). The lateral excretory vessels and nerves have changed places, those of the left now lying on the right and vice versa. The same arrangement persists in segment 2 (Fig. 18), and thereafter throughout the strobila.

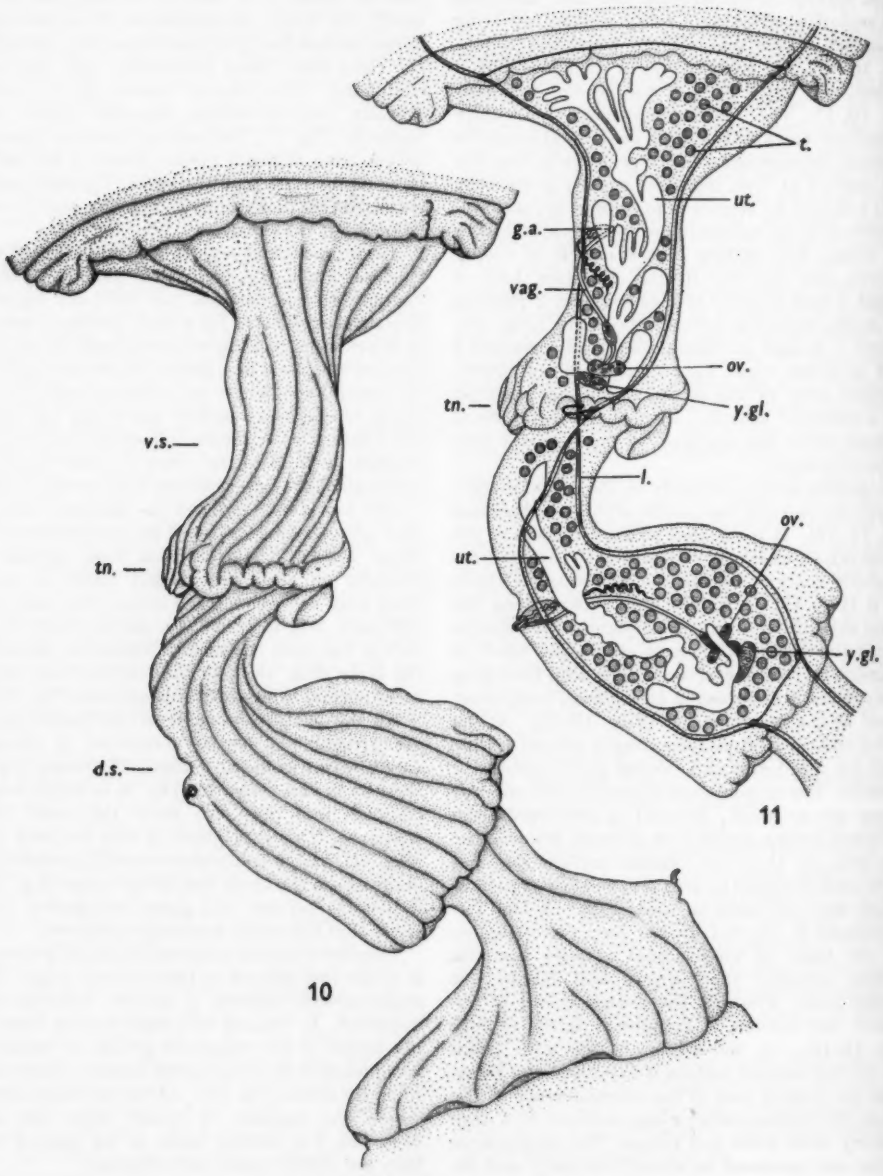
The reproductive organs, also, are affected by the torsion. From the mid-dorsal genital atrium the vagina and vas deferens run obliquely backwards along the dorsal side for a short distance, then turn at the margin, passing round ventrally to the lateral excretory vessels and nerves to come round on to the ventral side where they extend obliquely backwards towards the middle line (Figs. 11, 13). The vas deferens is very much coiled; the vagina as usual enlarges in front of the ovary to form the receptaculum seminis lying ventrally to the uterus (Fig. 11).

The testes are reduced in number. Anteriorly they are evenly distributed in a single row dorsally (Figs. 11, 12). Further back they become more irregular in arrangement and fewer in number. They may be two or three layers deep and may be obliquely oval in transverse section with the long axis in line with the path of extension, laterally, of the body (Fig. 15). At the posterior end they are more normal in size and arrangement (Fig. 17).

All the component parts of the female genitalia are present but depart somewhat in shape and arrangement from the normal. The ovary is greatly reduced in size (Figs. 11, 14). It is small, oval and compact and does not show the usual bilobed structure. The yolk gland is also reduced in size (Fig. 11), and in transverse section is pulled slightly obliquely in the same line as the testes (Fig. 15). It is just behind the yolk gland in segment 1 of the series that the spiral torsion is completed.

The uterus is quite abnormal in shape. The segment is in the late mature or early gravid stage, but the characteristic pattern of uterine branches is not apparent. It consists of a main branch running up the centre of the proglottid giving off lateral lobe-like branches in a haphazard manner where there is room for them (Fig. 11). At the anterior extremity where the segment is broad, there are several branches, but farther back, in the narrow region, they are rather sparse and irregular.

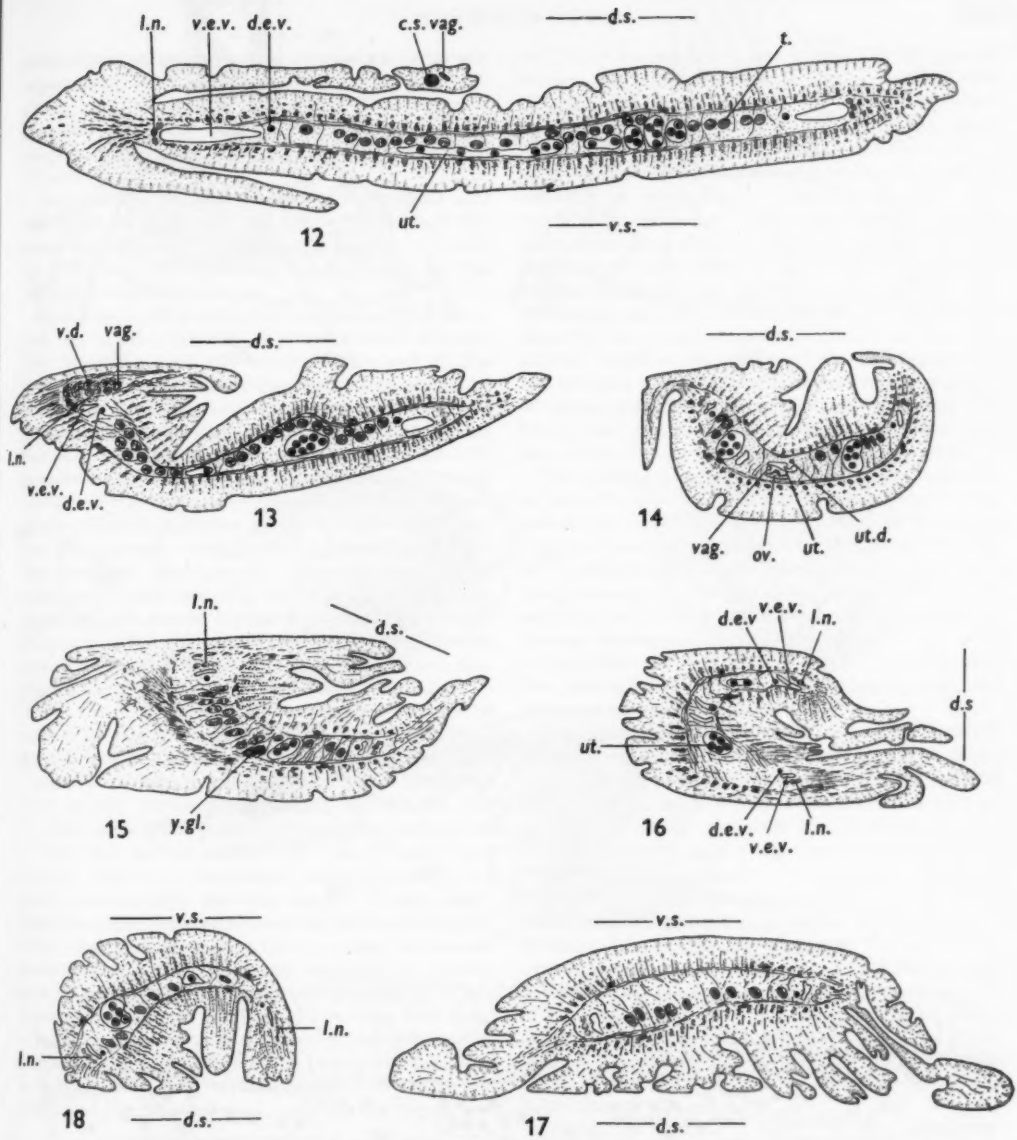
In segment 2 of the series the torsion is completed near the anterior end. The genital atrium, therefore, occupies its normal lateral position. Owing to the lengthening and narrowing of the segment, the vas deferens and vagina run obliquely backwards for a considerable distance (Fig. 11). The testes are



Figs. 10, 11. *Taenia taeniaeformis.*

Fig. 10. Three constricted segments of one of the specimens (specimen 1) involved in the knot showing spiral torsion in segments 1 and 2.

Fig. 11. Internal organs of the first two segments of specimen 1 showing the effect of spiral torsion.



Figs. 12–18. *Taenia taeniaeformis.*

Fig. 12. Transverse section through the first constricted segment of specimen 1 near the genital atrium showing the dorsal position of the terminal parts of the reproductive ducts.

Fig. 13. Transverse section a short distance behind Fig. 12 showing reproductive ducts turning around margin to pass ventral to lateral excretory vessels and nerves.

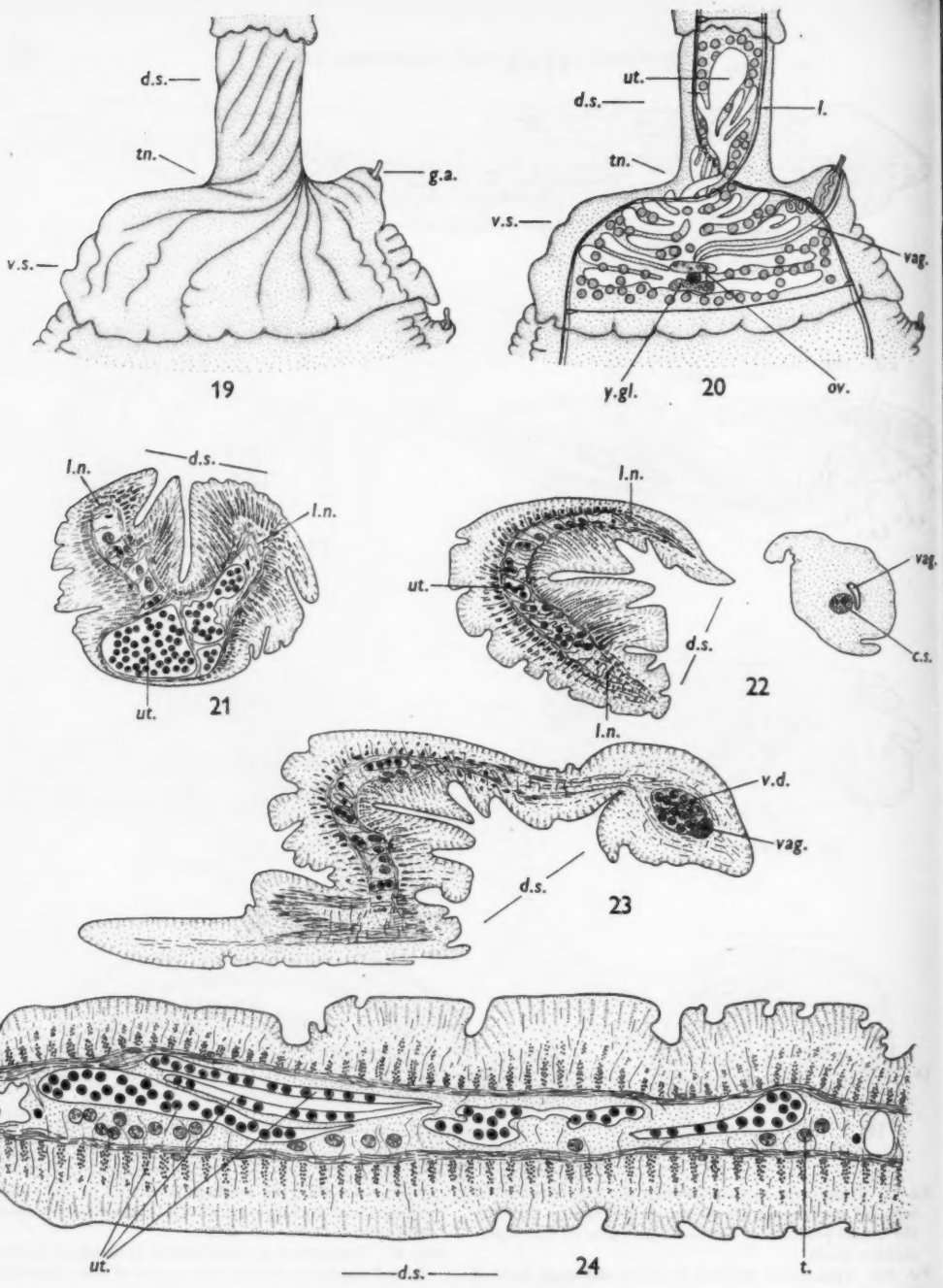
Fig. 14. Transverse section through the region of the reduced ovary.

Fig. 15. Transverse section through the region of the yolk gland showing increase in extent of the ventral surface and dorsal position of lateral excretory vessels and nerves of the left side.

Fig. 16. Transverse section behind yolk gland showing lateral excretory vessels and nerves of both sides lying one above the other.

Fig. 17. Transverse section showing completion of torsion, the dorsal surface now being ventral and the ventral dorsal.

Fig. 18. Transverse section through the anterior end of segment 2 of the series.



Figs. 19–24. *Taenia taeniaeformis.*

Fig. 19. Constricted segment (specimen 2) showing spiral torsion half way along the segment.

Fig. 20. Internal organs of the same segment showing the effect of spiral torsion.

Fig. 21. Transverse section through anterior half of same segment showing abnormal shape and uterus close to ventral body wall.

Fig. 22. Transverse section near genital atrium at point of torsion showing lateral excretory vessels and nerves of both sides lying one above the other.

Fig. 23. Transverse section through posterior half of segment showing lateral growth of segment to restore it to its normal width.

Fig. 24. Tranverse section through segment near posterior end.

reduced in number, especially anteriorly. The female organs again show a greatly reduced ovary and yolk gland, both being oval in outline. The uterus is restricted to a main stem running the length of the proglottid with a few irregular short branches given off laterally.

Segment 3 of the series broadens out rapidly and the following segments and those in front of the series are normal in appearance, showing no variations in the structure and arrangement of the contained organs.

Specimen 2. There are two constricted regions in this worm, each showing a spiral torsion through 180° involving both the surface layers and all the organs. The first of these two regions only will be considered. It comprises two segments, the torsion occurring in the second (Figs. 19, 20). The anterior half of this segment is very narrow, the posterior half broadening out rapidly. The torsion here takes place about half way along the segment. The anterior half of the segment is shown dorsal surface uppermost and the posterior half ventral surface uppermost. The cuticle is again deeply furrowed, the furrows taking a spiral course (Fig. 19), and the margins are duplicated in places and even in the two normal segments which follow and which separate the two constricted regions. The genital atrium is not displaced, being marginal throughout the strobila.

The effect of the torsion can be seen in surface view (Figs. 19, 20), and in a series of sections (Figs. 21–24). The anterior half of the segment is almost circular in outline in transverse section, the greater part of the circumference being formed by the ventral surface, the dorsal surface being reduced to a deep and narrow concavity. This is again not merely a folding of the worm, but the segment has grown permanently into this shape. A large sacciform branch of the uterus occupies the central region of the medulla and comes to lie very near the ventral body wall (Fig. 21). The muscle layers on the ventral side of the uterus are therefore considerably reduced. Elsewhere they are all present but have lost their normal arrangement owing to the folding of the body, the longitudinal muscle bundles being continued out into the folds. The lateral nerves and excretory vessels are in their normal position. At about the middle of the segment, just in front of the genital atrium, the torsion occurs, so that in surface view (Fig. 20) and section (Fig. 22) the lateral nerves and excretory vessels lie directly one above the other, those of the left side being dorsal and those of the right ventral. In the following wide region of the segment the widening is produced by growth outwards of lateral wings outside the excretory vessels and nerves (Fig. 23). Towards the posterior end the segment gradually flattens out completely, but the dorsal and ventral surfaces are now reversed (Fig. 24). The genital atrium is situated a little more posteriorly than in the normal segment, but, in spite of the greater width of the segment here, the vagina and vas deferens do not run straight towards the centre of the proglottid, but obliquely backwards (Fig. 20). The testes are fewer in number than usual and somewhat distorted in shape when seen in section.

The general arrangement of the female organs is not greatly affected. The uterus can be seen to turn over at the point of torsion (Fig. 20). The ovary and yolk gland are again greatly reduced in size and each is of a simple oval shape. The uterus consists of a main stem giving off few irregular branches in the anterior half; posteriorly the uterine branches are more numerous and may be duplicated or triplicated so that they are two or three layers deep (Fig. 24). This has not been observed in the normal segment and may be the result of torsion.

These two worms, therefore, specimens 1 and 2, which were knotted together, show each a spiral torsion in the parts involved in the knot. The effect of the torsion on the bodily structure can be seen by comparison with a normal strobila. The spiral torsion includes all parts of the segments concerned and is clearly visible externally. It is therefore different from the curious spiral torsion seen in the larval stage, and which is not apparent externally as it involves only the internal structures. The cause of the torsion in both cases is different. In the larval stage it presumably takes place early, in the growing zone of the strobila, due to some unknown cause, and is carried backwards, while in the present adult specimens it is localized and is the mechanical result of pressure produced by the twisting tightly of one worm around the other.

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Key to lettering of figures

(All drawings are semi-diagrammatic.)

c.	cirrus	*d.s.*	dorsal surface
c.c.	central chamber of Mehlis's gland	*g.a.*	genital atrium
c.s.	cirrus sac	*l.*	lateral nerves and excretory ducts
d.e.v.	dorsal excretory vessel	*l.m.*1	inner longitudinal muscles
d.n.	dorsal nerve	*l.m.*2	outer longitudinal muscles

l.n.	lateral nerves
m.gl.	Mehlis's gland
n.c.	nerve commissure
ov.	ovary
ovc.	ovicapt
ovd.	oviduct
p.gl.	prostate gland cells
r.c.s.	retractor muscle of cirrus sac
r.s.	receptaculum seminis
t.	testis
t.e.v.	transverse excretory vessel
t.m.	transverse muscles
tn.	point of torsion
ut.	uterus
ut.d.	uterine duct
vag.	vagina
vag.c.	cells surrounding vagina
vag.s.	vaginal sphincter
v.d.	vas deferens
v.e.v.	ventral excretory vessel
v.n.	ventral nerve
v.s.	ventral surface
y.d.	yolk duct
y.gl.	yolk gland

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ON THE SYNONYMY OF *MARSHALLAGIA ORIENTALIS* (BHALERAO, 1932) WITH *MARSHALLAGIA MARSHALLI* AND A RECORD OF ITS OCCURRENCE FROM THE MOUNTAINOUS REGION OF THE PUNJAB

By M. M. SARWAR, *Animal Husbandry Department, Sheikhupura, Pakistan*

In June 1946, one male and one female helminth of the species *Marshallagia marshalli* (Ransom, 1907) were collected from one of two goats (*Capra sibirica*), at Manali, which had been slaughtered soon after their arrival from Spiti (Kangra district, Punjab, India). The abomasa, small intestines, lungs and livers of both the animals were available for examination. The lungs, livers and small intestines did not yield any helminths, but one male and one female of *Marshallagia marshalli*, were collected from the abomasum of one goat, and a single male specimen of *Haemonchus contortus* was collected from the abomasum of the other goat.

Mönnig (1940) found *Marshallagia marshalli* only in desert areas in South Africa. Spiti presents the arid features of a desert, but the average elevation of mountainous ranges there is over 18,000 ft. The mean elevation of the villages is 12,000 ft. and it rises as high as 14,000 ft. There is severe frost, but snow does not exceed a depth of 2½ ft. in the winter. In the summer the sun is very powerful in this treeless and shadeless area and there are only slight rains in July and August, the annual rainfall being only 2 in. Geologically this region is connected with Tibet.

The author examined Bhalerao's (1932) collections of *M. orientalis* at Izatnagar and found that his descriptions correspond with that of *M. marshalli*. Investigation of the points of differentiation advanced by Bhalerao (1932) revealed that the spicules under pressure of a cover-slip assumed the form drawn by Mönnig (1940). The largest process of each spicule is pointed, while the other two processes have bifid and square ends respectively.

The eggs in Bhalerao's specimens fell within the size-range of Mönnig (1940) and their appearance was characteristic.

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DESCRIPTION OF TWO NEW SPECIES OF *ASCODIPTERON* FROM AFRICA AND ONE SPECIES OF *NYCTERIBOSCA* FROM MADAGASCAR (DIPTERA, STREBLIDAE)

By B. JOBLING, F.R.E.S.

From the Wellcome Laboratories of Tropical Medicine, London

(With 4 Figures in the Text)

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The description of one new species of *Ascodipteron* and another one of *Nycteribosca* is based on the material collected in Africa and Madagascar respectively by Mr H. Hoogstraal, of the United States Naval Medical Research Unit No. 3. These specimens represent a part of the collection which was sent to me for identification by Mr R. L. Wenzel of the Chicago Natural History Museum. In this paper I have also included the description of another new species of *Ascodipteron* of which two specimens were collected by Mr T. S. Jones in West Africa.

As the specific characters of the species of the genus *Ascodipteron* have not been properly understood, they are described here in the comparative form, together with the morphology of the head, thorax and the abdomen.

THE SPECIES OF THE GENUS *ASCODIPTERON* ADENS., AND THEIR SPECIFIC CHARACTERS

The first described species, *Ascodipteron phyllorhinae* Adensamer (1896), which was found on a bat of the genus *Phyllorhina* (=*Hipposideros*), from Java, cannot be recognized from the original description. It is likely that its identity will never be established, because the type was sectioned by the author, and therefore must be regarded as lost.

In 1898 Monticelli introduced the second species, *Ascodipteron lophotes*, which was found on *Rhinolophus clivosus* Cretzchmar, from Eritrea. He dissected his two specimens, and described their morphology and anatomy. For the genus he created a new family, Ascodipterinae, but omitted to give a separate diagnosis of his species. At present it is impossible to state by what characters this species differs from the other African species; but as the paper of Monticelli is illustrated by many drawings, there is a possibility that its identity could still be established if new specimens of *Ascodipteron* are collected from the same host in Eritrea, and their structure is compared with that shown in the drawings.

The descriptions of *Ascodipteron siamense* Speiser (1903), from *Hipposideros bicolor* Temminek, from Biserat in Jalor, Siam, Malay Peninsula, and that of his other species, *Ascodipteron tabulatum* (1908), from *Miniopterus* sp., from Madagascar, are based upon the position of the abdominal spiracles, which, however, is not a reliable character. Dr O. Theodor of Palestine informed me that during his recent visit to several museums in Western Germany and to that of Vienna, he has not been able to find Speiser's types, although he was specially looking for them. It is possible that they are still preserved in Eastern Germany.

In the description of *Ascodipteron emballonurae* Banks (1911), there is indicated one significant character—the presence of the branch near the tip of the arista of the antennae. The drawings illustrating the paper may also help the identification of this species, especially those which show the structure of the posterior end of the abdomen of the female, the theca of the labium and the parts of the leg. The type is probably preserved in the American National Museum, where it was found on *Emballonura* sp., from Borneo.

In 1912 Muir described in the same paper the following two species: *Ascodipteron speiserianum*, from *Miniopterus schreibersi blepotis* Temminek, from Amboina, and *Ascodipteron australianse*, from the same host, from North Queensland. As he could obtain an almost unlimited number of the specimens of *A. speiserianum*, he studied its morphology and anatomy, gave the first description of the development of these flies, and also included a separate

diagnosis for the male and the female. Many years ago I asked Dr Muir about the types of his species, but he had apparently forgotten where they had been deposited, and could not give me a definite answer. However, he kindly presented me with some of his microscopical preparations of this species, which are in my collection. Of these preparations only those of the wing and the leg are in good condition, whereas all the others have badly mounted fragments of the different parts of the body and are unsuitable for a study of the specific characters. Recently the paratypes of this species have been found by Dr O. Theodor, in the collection of the British Museum (Natural History), and therefore the identity of *A. speiserianum* can be established.

The description of Muir's other species, *A. australianse* is very brief. He states that it 'differs from *A. speiserianum* in the following points: head and thorax smaller and darker, notum more convex and hairy, spines rounded, the exposed posterior part of abdomen stouter and shorter'. At present the systematic position of this species is not clear. It could be established if the specimens of *Ascodipteron* collected from *Miniopterus schreibersi blepotis* in the type locality are compared with the above description and with *Ascodipteron speiserianum*.

The last species, *Ascodipteron africanum*, was described in my two papers (1939, 1940). The first paper gave the description of the female, which is based upon the detailed study of the head and the thorax, while the second paper gives the description of the male. The gynotype and the androtype, with many paratypes, are present in the British Museum (Natural History), and the two paratypes are in my collection.

An examination of the females of the two new forms, which are described in this paper, and their comparison with *A. africanum*, shows that the species of this genus have many distinctive characters by which they can be easily separated from each other.

The external morphology of these flies has already been described in my paper (1939); here it is given in abbreviated form. In this description the terminology of some parts of the thorax and the abdomen is altered according to my recent investigation of their homology.

The abdomen. In the female dissected out of the skin of the bat, it is possible to see only the greatly modified abdomen, because the other regions of the body, such as the head and the thorax lie concealed in its chamber. The abdomen consists of the large, pyriform or cucumiform anterior part and the small knob-shaped or mushroom-shaped posterior part (Fig. 1 A). It has been found during the preparation of this paper that the anterior part is formed by the greatly hypertrophied first abdominal segment, while the posterior part is formed by the fusion of all the other segments. The posterior part bears the concentric rows of small setae, which represent the remains of the marginal setae of the abdominal segments. In the middle there is the slit-like aperture of the shallow pocket-shaped depression, into which open the rectum and the vagina. Dorsally to this aperture there are present the cerci (Jobling, 1951), and above them and more or less close together the spiracles of the sixth abdominal segment. Laterally, or slightly dorso-laterally, to each of these spiracles lie the spiracles of the fifth segment, while each spiracle of the seventh segment lies latero-ventrally to the slit-like aperture.

The structure of the abdomen shows the following differences in the species of this genus: in *A. africanum* Jobling the anterior part is pyriform and the posterior part is small and knob-shaped. The cerci are ring-shaped and quite distinct, with many very fine setae. There are no setae on the posterior end between the aperture and the sixth abdominal spiracles. In *A. rhinopomatos* n.sp. the anterior part is cucumiform; the posterior part is large and together with the narrow part, which connects it with the anterior part, is mushroom-shaped. The cerci are very small, irregularly shaped sclerites, each with two long and four very minute setae (Fig. 1A, *c*). From *A. africanum* this species differs also by the presence of a few setae between the aperture and the sixth abdominal spiracles. In *A. jonesi* n.sp. both parts of the abdomen were distorted and were unsuitable for comparison. In this species there are no setae between the aperture and the sixth abdominal spiracles. The cerci are more or less fan-shaped, with three long and four or five very minute setae (Fig. 2D).

For the examination of the head and the thorax, they must be dissected out of the chamber which is formed by the hypertrophied first abdominal segment. In order to see them during the dissection, the specimen should be boiled in potash and cleared in clove oil. The posterior end of the abdomen must be cut off before the boiling, because it is very difficult to separate it from the rest in the softened specimen without distortion. If there are present two specimens of the same species, one of them should be mounted dorsally and the other ventrally. A single specimen must always be mounted in the lateral position, after examination of the dorsal and ventral parts of its head and thorax. All the parts of the same specimen must be mounted on the same slide, but under different cover-slips. It is necessary to support the cover-slip with three pieces of glass or cellophane, otherwise the specimen is liable to be distorted by pressure and rendered useless for study.

The head and the proboscis. The head of these flies is not a sclerotized capsule as is that of the other Diptera. Its largest portion is formed by the greatly developed membrane, of which the anterior part envelops the basal part of the theca of the

labium and the posterior part overlaps the anterior part of the thorax (Figs. 1 G and 2 E). The dorsal part of the head is occupied by the frons (*f*) and the latero-vertices (*lv*). The frons can be divided into an anterior and a posterior part. The anterior part resembles the outstretched wings of a bird when examined dorsally, while the posterior part forms the antennal pits in which lie the two segmented antennae. The clypeus (*cl*), as in all the other Streblidae, is divided into two small parts which are attached to the anterior margin of the frons (Fig. 1 B). Inside the head the clypeal parts are connected by the apodemes with the cibarial pump, forming the stirrup-shaped fulcrum (*fu*). Under the posterior parts of the latero-vertices lies a very small sclerite, which in my previous paper was regarded as the prothorax, whereas in fact it represents the dorsal part of the occiput (*o*). On each side of the head, close to the antero-lateral margin of the thorax and the coxa of the foreleg lies the genae (*g*). The ventral part of the genae merges into the narrow postgenae, which occupy the ventral position and lie close to the prosternum of the thorax.

The most conspicuous part of the proboscis is an enormously developed theca of the labium (*th*), inside which lies the rod-shaped, cylindrical labial gutter (Fig. 2 E, *lg*). The anterior margin of the labial gutter is connected with that of the theca by the membrane, which bears fourteen rows of strongly sclerotized teeth, while its posterior end is articulated to the Y-shaped labial vinculum (Fig. 2 E, *v*), which represents the modified posterior wall of the theca.

With the exception of the occipital sclerite, the teeth of the labella and the fulcrum, all the other parts of the head are very easily observable in properly mounted specimens, and have clear-cut characters. As regards the teeth of the labella, their appearance changes according to their position. They are probably of more or less the same structure in different species, but this is very difficult to see, unless the blades are dissected and mounted in the same position. In the specimen shown in Fig. 1 G, the teeth are fully protracted, while in that shown in Fig. 2 E, they are retracted and have an entirely different appearance. In the last specimen the long dorsal teeth are broken off, which often happens during the dissection of these flies from the skin of the bat.

The thorax. In all the specimens which have been examined, the thorax is more or less strongly compressed, and is higher than long. Owing to the absence of the transverse suture, the mesonotum is formed by the large prescutoscutum and the small scutellum. Behind the scutellum is the tergite of the metanotum (*met*) which is regarded by the morphologists as the postscutellum. At each side of the prescutelar part of the mesonotum lies the notopleuron (*np*), which in these flies is more distinct when the thorax is examined dorsally. The pronotum consists of two parts: the antepronotum and the postpronotum. The antepronotum (*ap*) is arch-shaped and is fused with the anterior margin of the prescutum, while the postpronotum consists of the flat upper part and the humeral callus (*hc*). In *A. rhinopomatos* the upper part of the postpronotum is separated from the mesopleuron (*mes*) by a very fine suture which extends ventrally to the anterior or mesothoracic spiracle (*as*). The mesopleuron or anepisternum is a very distinct sclerite, which is separated from the notopleuron by the narrow horizontal area and from the pteropleuron or anepimeron (*p*) by the wide pleural membrane. As in the species of *Ascodipteron* the structure and the chaetotaxy of the mesopleuron and the pteropleuron are different; they are important taxonomically. Between the pteropleuron and the metanotum lies the pleurotergite (*ptg*), which extends ventrally as far as the stump of the halter (*sh*). It is not well defined in all the species and is not separated by the suture from the region which lies below the halter and posteriorly to the posterior or metathoracic spiracle (*ps*). It is possible that this region represents the episternum, while the sclerite, which overlaps the coxa of the hind leg and extends to the base of the coxa of the middle leg, is probably formed by the epimeron and by the meron (*mer*). In this highly specialized genus it is very difficult to homologize the parts of this region of the thorax with those of the other higher Diptera. In my previous paper (1939), the part which is designated here as the meron, was called the hypopleuron. But it is more likely that the region of the hypopleuron or katepimeron (*h*) lies ventrally to the pteropleuron. The sternopleuron or katepisternum (*st*) in this genus is greatly reduced, but is quite distinct. Its anterior margin, as in the other Streblidae, is connected by the internal apophysis (shown by dotted lines in Fig. 1 G) with that of the pronotum.

The ventral wall of the thorax differs from that of the other Streblidae by the presence of a well-developed prosternum (*pst*) or basisternum of Crampton (1942), which lies between the coxae of the fore legs. The next sternal region belongs to the mesothorax. It is probably composed of the basisternum and the furcasternum, of which the inrolled parts form the median ridge. The furca of the mesosternum is shown in Fig. 1 G by dotted lines under the hypopleural region. For descriptive purposes this region is called here the mesosternum (*ms*). In the other Streblidae it was called by me the sternopleura, the term adapted from Young (1921). The metasternum is a more or less rhomboidal region which lies between the coxae of the middle legs. It is probably also composed of the basisternum and the furcasternum. In my previous paper, where I

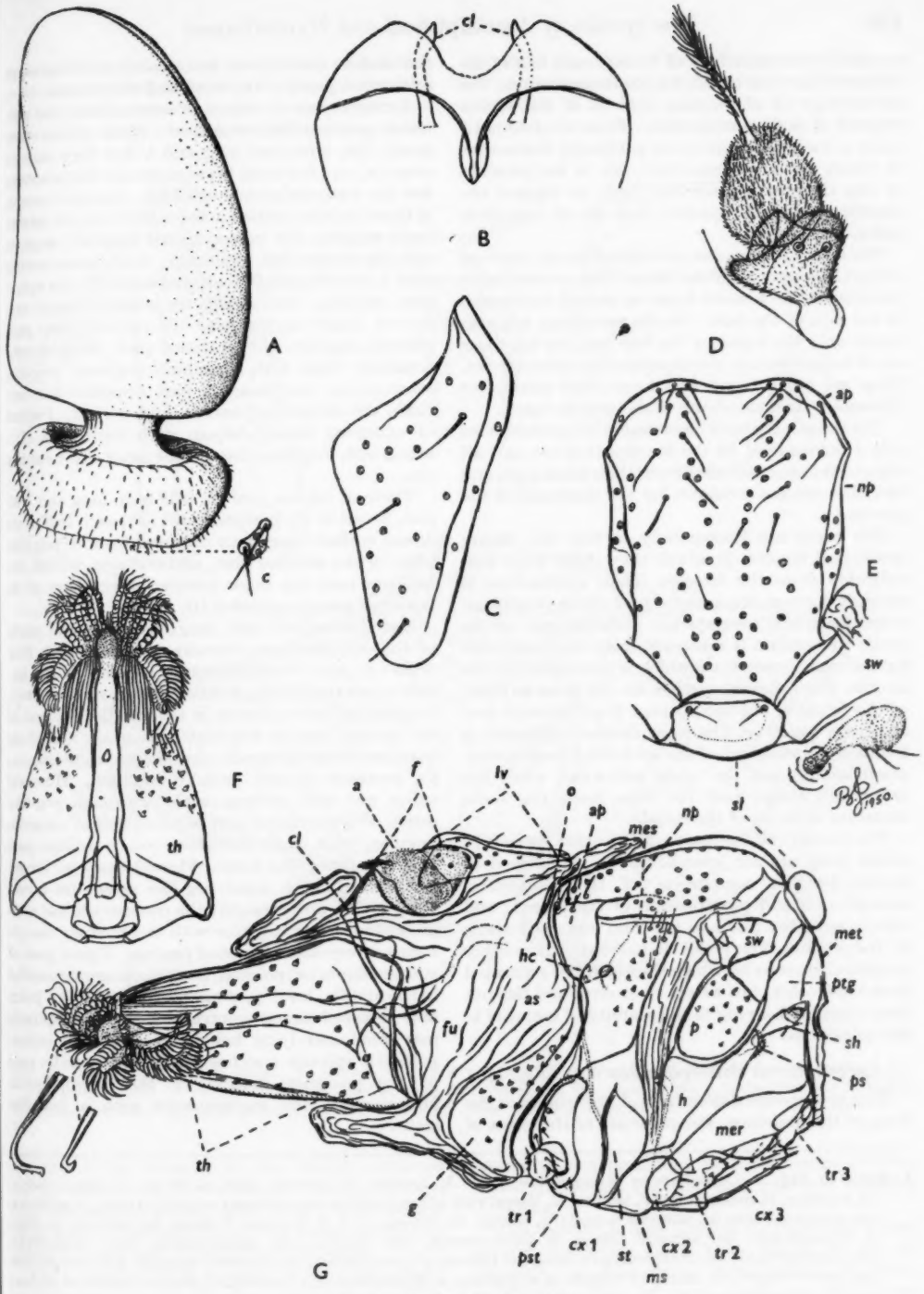

Fig. 1. For legend see p. 130.

adapted the terminology of Young, each half of the metasternum was called the pleurotrochantin. The morphology of the sternal regions of the higher Diptera is still controversial. From a systematic point of view they have good subfamily characters, of which the most important one is the presence of the distinct prosternum; but, as regards the classification of the species, they are of very little value.

The legs of the females are cast off at the junction of the trochanter with the femur. This process takes place when the females begin to embed themselves in the skin of the bats. Of the remaining two segments only the coxae of the fore and the hind legs are of importance in the classification of the species. They are much larger and have more distinctive characters than the coxae of the middle legs.

The wings have been described in *A. speiserianum* and *A. africanum*. In the females they are cast off close to the articular sclerites of their basal part, and therefore are not available for the diagnosis of the species.

The males are known only in the two above-mentioned species, in which they differ very considerably from the females. Their mesonotum is strongly convex; the anterior part of the prescutum is produced and overlaps the posterior part of the head; the scutum is separated from the prescutum by the transverse suture which is incomplete in the middle. Their pleural sclerites are the same as those of the females, but are without peg-like setae and are closer together. The most distinct difference is found in the structure of the sclerotized head, a very small labium and the ovoid abdomen; while the antennae, wings and the legs have the same structure as those of the female.

The identity of the males of the other species, in which they are not known, can be established in future by the comparison of their antennae, mesopleuron and pteropleuron of the thorax, and the coxae of the fore and the hind legs, with those of the females. The males undoubtedly have other specific characters by which they could be separated from each other, but as yet their structure has not been compared, owing to the scarcity of material in the collections.

Ascodipteron rhinopomatos n.sp. (Fig. 1)

This new species differs from *A. africanum* in the form of the abdomen, the structure of the theca of the labium, mesonotum and in almost all the other sclerotized parts of the head and the thorax.

Anterior part of abdomen cucumiform, the posterior part mushroom-shaped. Each cercus very small, flat, with two long and a few very minute setae (A, *c*); a few setae between the slit-like aperture and the sixth abdominal spiracles. Anterior margin of frons broadly excised in the middle (B); its lateral parts angular, the posterior part narrowly angular and slightly swollen at the tip. Each latero-vertex with a few irregularly scattered setae (C); its apical part angular, with a slightly concave outer and convex inner margin near the tip; its inner part rotundo-angular, the posterior part being evenly rounded. Gena with irregularly scattered peg-like setae (G, *g*); its broadest part opposite the fore coxa; the dorsal part narrow and curved. Pedicel of antennae funnel-shaped, with two setae (D); arista with long branches on the inner side near the tip.

Theca of labium conical, 0·39 mm. long and 0·31 mm. broad in the posterior part (F); each half of its dorsal surface bears four diagonal rows of peg-like setae in the anterior part, and one seta behind the last row near the inner margin; upper part of its posterior margin rounded (G, *th*).

Mesonotum 0·37 mm. long; antero-lateral parts of the antepronotum broadly rounded, with four setae (E, *ap*). Prescutoscutum broader in the anterior part than in the middle, with two interrupted, longitudinal rows of setae in the middle; the rest of its surface bears a few scattered setae. Scutellum with two setae far apart near the anterior margin; its posterior margin broadly rounded. Humeral callus not well defined, with two small peg-like setae. Postero-dorsal part of mesopleuron rotundo-angular, with small fine setae (*mes*); middle part with small peg-like setae. Pteropleuron (*p*) longer than broad, with small peg-like setae; its dorsal margin thin and straight; the postero-ventral margin rounded and together with the anterior margin forms a very short, pointed process. Upper part of sternopleuron (*st*) pointed; posterior margin rounded in the middle; lower part narrowed almost to a point and directed antero-ventrally. Anterior spiracle (*as*) small and lying behind the humeral callus; posterior spiracle (*ps*) lies close to the middle part of the posterior margin of the pteropleuron. Mesosternum and metasternum with a few fine setae.

Legend to Fig. 1. *Ascodipteron rhinopomatos* n.sp. A, female; B, anterior part of frons; C, latero-vertex; D, antenna; E, mesonotum; F, labium; G, lateral view of head and thorax (different magnifications). *a*, antenna; *ap*, antepronotum; *as*, anterior spiracle; *c*, cercus; *cl*, clypeus; *cx* 1, 2, 3, coxae; *f*, frons; *fu*, fulcrum; *g*, gena; *h*, hypopleuron; *hc*, humeral callus; *lv*, latero-vertex; *mer*, meron; *mes*, mesopleuron; *met*, metanotum; *ms*, mesosternum; *np*, notopleuron; *o*, occipital sclerite; *p*, pteropleuron; *ps*, posterior spiracle; *pst*, prosternum; *ptg*, pleurotergite; *sh*, stump of haltere; *sl*, scutellum; *st*, sternopleuron; *sw*, stump of wing; *th*, theca of labium; *tr* 1, 2, 3, trochanters.

Coxa of the fore leg (*cx* 1) with four or five thin setae; its upper part rounded and curved posteriorly; the lower one rounded in the posterior part and more or less angular in the anterior part; posterior margin almost straight in the middle, anterior margin convex near the trochanter. Coxa of the hind leg (*cx* 3) slightly longer than broad, with straight inner margin and slightly curved outer margin; its chaetotaxy resembles that of *A. africanum*.

The gynotype and one paratype have been found on *Rhinopoma cystops* Thomas, near Sakkara, Giza, Egypt, 20 May 1950 by Mr H. Hoogstraal. The other two paratypes were collected by him from *Rhinopoma cystops*, no. H.H. 5597, 2 km. from Giza Pyramids, Egypt, 4 June 1950. The genotype and the two paratypes are preserved in the Chicago Natural History Museum, and the other paratype is in my collection.

This species is also present in Palestine, where several specimens have been found by Dr O. Theodor, on *Rhinopoma microphyllum* Geoffroy, in Tiberias. Two of these specimens have been

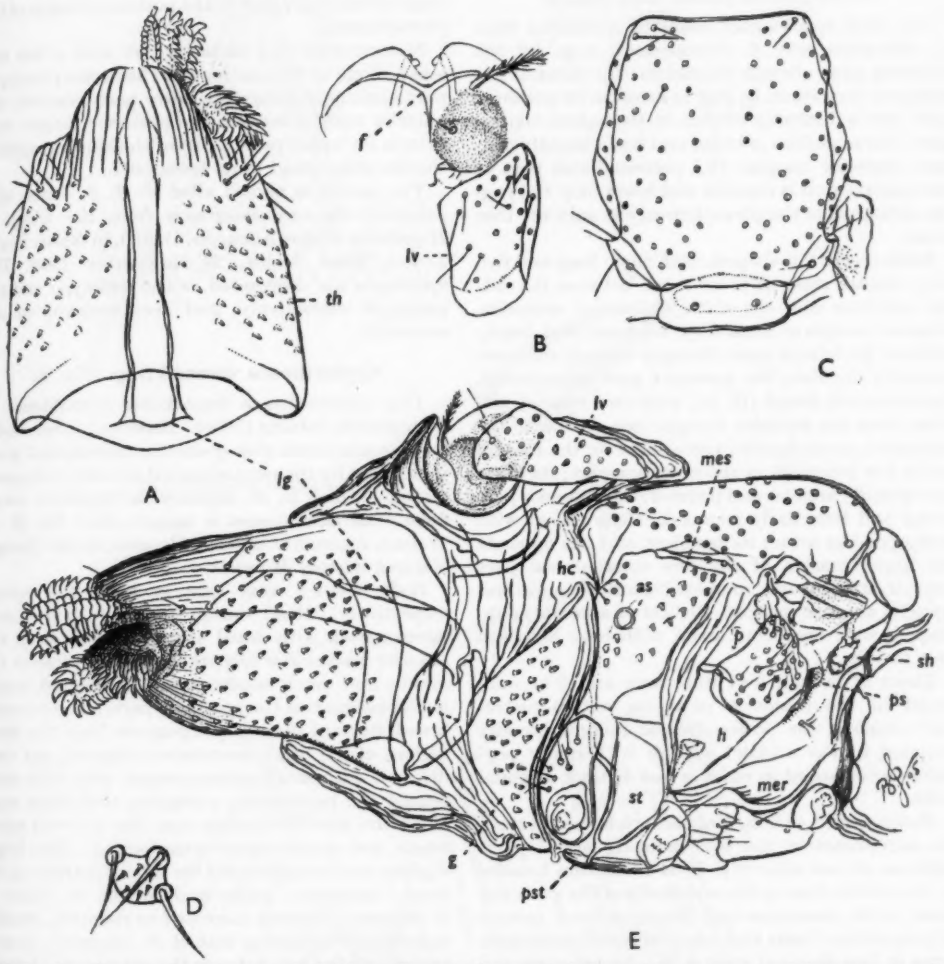

Fig. 2. *Ascodipteron jonesi* n.sp. A, labium; B, anterior part of frons (*f*), antenna and latero-vertex (*lv*); C, mesonotum; D, cercus; E, lateral view of head and thorax (A, B, C and E same magnification; D, higher magnification). *lg*, labial gutter; *v*, labial vinculum; other lettering as in Fig. 1.

compared with the gynotype, and are designated as paratypes. One of them is preserved in my collection, and the other one is in that of Dr O. Theodor.

Ascodipteron jonesi n.sp. (Fig. 2)

This new species can easily be separated from *A. africanum* and *A. rhinopomatos* n.sp. by the following more obvious characters: the theca of the labium is very stout, as long as broad in its posterior part, and is distinctly swollen in the antero-ventral part. The scutellum is broad and has a slightly concave posterior margin. The postero-dorsal part of the mesopleuron is angular and bears only the peg-like setae, while the pteropleuron has only the thin setae.

Each cercus fan-shaped, with three long and five very minute setae (D); no setae between the slit-like aperture and the sixth abdominal spiracles. Anterior margin of frons (B, *f*) narrowly and deeply excised; its lateral parts strongly curved and very narrowly angular; the posterior part acuminated. Latero-vertex broad (B, *lv*), with two rows of fine setae near the anterior margin, one row near the thickened outer margin, three setae on the margin and a few scattered in the posterior part; its inner part broadly angular and pointed; the posterior part broad and irregularly rounded. Gena (E, *g*) with many peg-like setae; its broadest part lies opposite the upper margin of the fore coxa; dorsal part angular, with broadly rounded posterior margin and straight anterior margin. Pedicel of antennae cup-shaped, with one seta; arista with long branches near the tip.

Theca of labium 0·41 mm. long and 0·41 mm. broad in the posterior part; its antero-ventral part swollen; the postero-lateral margin broadly rounded in the middle; surface with many peg-like setae arranged in more or less distinct diagonal rows.

Mesonotum 0·46 mm. long; antero-lateral parts of the antepronotum not well defined; lateral parts with one or two setae (C). Prescutoscutum broader in the middle than in the anterior and the posterior parts, with transverse and broad anterior margin (C); its surface bears fine setae, of which some form more or less diagonal rows in the lateral parts and two longitudinal rows in the middle of the prescutelar part. Scutellum broad, with slightly concave posterior margin, and with two setae far apart in the middle. Humeral callus (E, *hc*) distinct, with two fine setae. Postero-lateral part of mesopleuron angular, pointed and with only peg-like setae; four peg-like setae below the anterior spiracle. Pteropleuron as long as broad, with many fine setae; its upper part more or less rotundo-angular; the postero-ventral margin straight, the antero-dorsal margin slightly concave. Upper part of sternopleuron thin, with indistinct apex; the posterior margin thin and straight in the middle; the anterior margin thick in the lower part and slightly concave; lower part rounded and ends in a thin curved process. Anterior spiracle large, ring-shaped; posterior spiracle lies close to the upper part of the posterior margin of the pteropleuron.

Mesosternum and metasternum with a few fine setae. Coxa of the fore leg with fine setae; its upper part slightly angular; the lower part rounded; the anterior margin convex, the posterior margin concave in the upper part. Coxa of the hind leg slightly shorter than broad in its basal part.

The species is named after Mr T. S. Jones, who collected the two specimens from the wings of *Hipposideros abae* Allen, no. B. 150, in Njala, Sierra Leone, West Africa, 24 September 1949. The specimens are designated as the genotype and the paratype respectively, and are preserved in my collection.

Nycteribosca wenzeli n.sp. (Fig. 3)

This species has a remarkable resemblance to *N. bequaerti* Jobling (1936). However, it can easily be separated from this species by its elliptical post-vertex and by the semi-spherical seventh abdominal sternite, which in *N. bequaerti* is distinctly cordiform. The new species is named after Mr. R. L. Wenzel, Assistant Curator of Insects, at the Chicago Natural History Museum.

Body 4·0–4·4 mm. long in male and female respectively. Head darker than the body; each latero-vertex with usual very strong setae in the anterior part, a few moderately strong setae in the middle and much smaller setae of different length in the lateral and the posterior parts; medio-vertex broad, but not distinctly separated from the other regions of the head; postvertex elliptical, not very distinct, with small setae; occiput with thin setae behind the postvertex; postgenae with long setae along the anterior margin near the rostrum membrane, and much smaller setae behind. Eyes large, slightly projecting beyond the lateral margins of the head. Antennae, palps and labium as those of *N. bequaerti*. Thorax more or less rounded, broader than long, resembling that of *N. bequaerti*; prescutum with a few long setae in the antero-lateral parts, and with slightly shorter setae along the lateral margins and near the transverse suture; scutum with long setae along the lateral margins, and with a transverse patch of similar setae in the posterior part; scutellum broadly rounded, with many long setae. All setae of the head and the thorax distinctly shorter than those of *N. bequaerti*. Thoracic squamae (Jobling, 1951) broadly rounded. The ventral surface of the thorax flat with uniform small setae. Legs normal; dorsal surface of femora with strong setae, the ventral surface with small setae.

Fig. 3.
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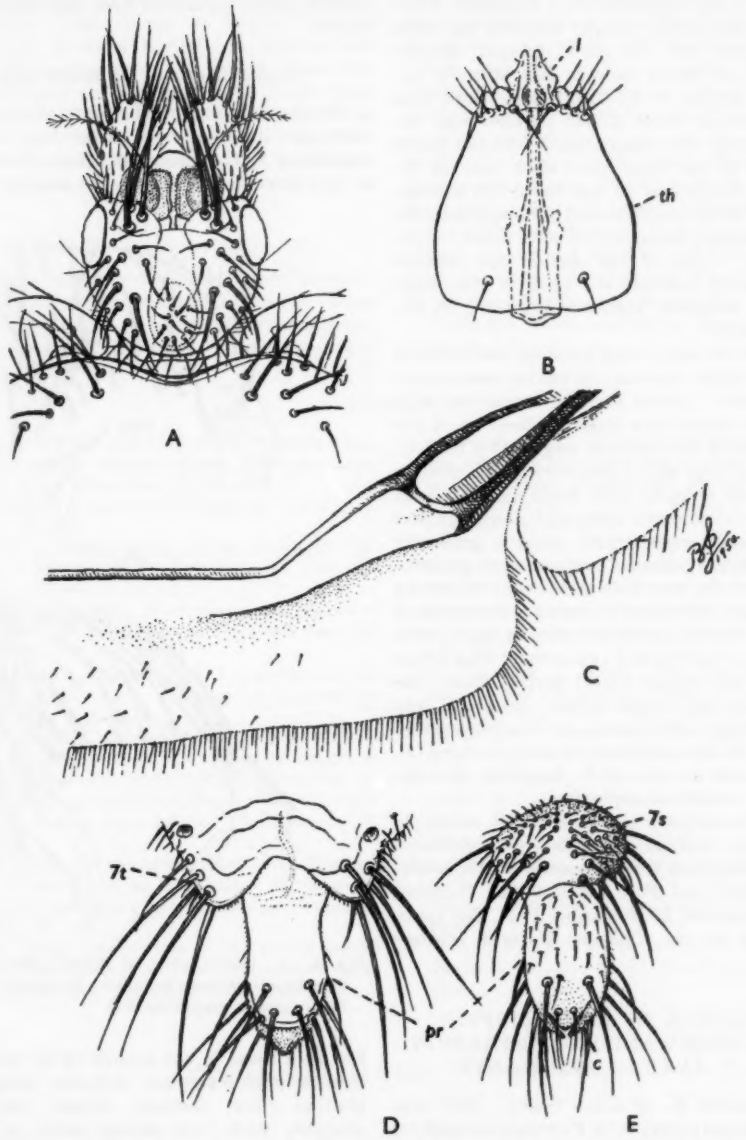

Fig. 3. *Nycteribosca wenzeli* n.sp. A, dorsal view of head; B, labium; C, basal part of wing, showing bend in fifth longitudinal vein; D, dorsal, and E, ventral view of posterior end of abdomen of female. *l*, labella; *th*, theca; *c*, cerci; *pr*, proctiger; 7*s*, seventh sternite; 7*t*, seventh tergite (A, C, D and E, same magnification; B, higher magnification).

Wings 4·1–4·5 mm. long in male and female respectively, resembling those of *N. bequaerti*. First longitudinal vein nearly straight between the bend in its basal part and the costal margin; second longitudinal vein bends more or less strongly towards costal margin in its distal part; third and fourth longitudinal veins almost parallel near the apex of the wing; fifth longitudinal vein has much sharper bend in its basal part than that of *N. bequaerti* (C); distal half of first basal cell oblong, with parallel sides; marginal and submarginal cells with many setulae; humeral cell with much longer setulae, while those of the costal cell become gradually shorter towards the apex of the wing. Alula broadly rounded. Halteres presenting no distinctive character.

Abdomen with many long setae on each side of the bare part of the dorsum; its venter bears many very small setae. Second sternite triangular, with slightly longer setae than those on the rest of the venter. Lobes of the seventh tergite (7*t*) conical, with five very long and a few very much shorter setae. Seventh sternite (7*s*) semispherical, with slightly incurved posterior margin; its anterior part bears very small setae, which become gradually longer towards the posterior part, while its posterolateral part and the lateral margins bear very strong setae. Proctiger (*pr*) slightly narrower than that of *N. bequaerti*, constricted in the anterior part, with four dorsal, four lateral and two ventral long setae; the anterior half of its dorsal surface bare; the ventral surface with small setae; one small seta between the long ventral setae, and two moderately long setae on the subanal plate between the cerci (*c*).

Male abdomen as that of *N. bequaerti*, but the hypopygeum is more semispherical.

Two specimens were examined: the holotype, female; and the allotype, male. They were collected by Mr H. Hoogstraal from *Rousettus madagascariensis* Grandidier, at Fort Mananteina, Dauphin district, Madagascar, 29 November 1948. The types are preserved in the Chicago Natural History Museum.

RESTORATION OF *PTERELLIPSIS PROXIMA* SÈGUY AND ITS COMPARISON WITH *P. ARANEA* COQUILLETT

For many years *P. proxima* Sèguy, 1927 was regarded by me as a variety of *P. aranea* Coquillett, 1899, and in my paper (1949) it was made a synonym of the latter. Recently, Mr R. L. Wenzel has very kindly presented me with the specimen of this species, which was collected near Therezopolis, Rio, Brazil. An examination of this specimen shows that it differs from *P. aranea* by many characters and fits the description of the species of Sèguy. Therefore, *P. proxima* must be regarded as a distinct species.

The most obvious characters by which these two species can be separated from each other are shown below.

Pterellipsis aranea Coquillett (Fig. 4 A)

Mesonotum very broadly rounded when examined laterally; its transverse suture very distinct and extending to the lateral margins. Prescutum with a very few strong setae in the anterior part and a few thin setae in the middle of its posterior part. Scutum with six setae. Anterior margin of mesopleuron (*mes*) distinct; dorsal margin almost straight, with four strong setae, of which the posterior one is the strongest; upper part of the posterior margin rounded, and together with the dorsal margin forming short, triangular process. One strong seta near the anterior spiracle (*as*). Wings 0·8 mm. long and 0·14 mm. broad. Femora of the fore legs 0·68 mm. long and 0·22 mm. broad. Femora and tibia of the hind legs of the same length, measuring 1·7 mm.

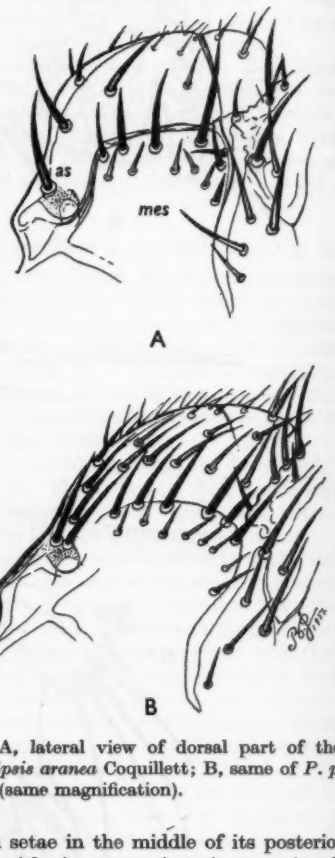

Fig. 4. A, lateral view of dorsal part of thorax of *Pterellipsis aranea* Coquillett; B, same of *P. proxima* Sèguy (same magnification).

The species parasitizes *Artibeus jamaicensis jamaicensis* Leach, in Canal Zone and Panama; *A. planirostris polax* Peters, in British Guiana; *A. planirostris trinitatis* K.And., in Tobago and Trinidad; *Phyllostomus* sp., in Brazil. Recorded from unknown species of bats in Cuba, Porto Rico, West Indies and Venezuela. The following records are given by Hoffmann Mendizabal (1944): from *Artibeus jamaicensis jamaicensis* Leach, in Mexico; and from *A. jamaicensis yucatanicus* Allen, in Yucatan.

Pterellipsis proxima Sèguy (Fig. 4 B)

Mesonotum humpbacked when examined laterally; its transverse suture not well defined in the lateral parts. Prescutum with strong setae in the anterior and the lateral parts, and with triangular patch of very thin setae in the middle of the posterior part. Strong anterior setae form two diagonal rows, which extend from the antero-lateral corners of the prescutum almost to the middle of the median suture. Scutum with four small setae near the transverse suture, and six small setae in the posterior part. Anterior margin of mesopleuron indistinct; postero-dorsal part rounded; dorsal margin slightly convex, with six strong setae, of which the fifth is very much shorter than the others. Two strong setae near the anterior spiracle. Wings of the same length as those of *P. aranea*, but 0·22 mm. broad. Femora of the fore leg 0·60 mm. long and 0·24 mm. broad. Femora and tibiae of the hind legs of the same length, each measuring 1·4 mm.

Recorded from unknown species of bats, in Argentina and Brazil.

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TWO NEW MITE PARASITES OF NATAL BATS (LISTROPHORIDAE, SARCOPTIFORMES)

By R. F. LAWRENCE, Ph.D.
Natal Museum, Pietermaritzburg, South Africa

(With 4 Figures in the Text)

Three species of Listrophorid mites representing two genera, *Alabidocarpus* and *Labidocarpus*, are known from the European horse-shoe bat *Rhinolophus ferro-equinum*, and one species of *Alabidocarpus*, *A. nasicolus*, has already been described (1938) from the closely related Natal horseshoe bat *Rhinolophus geoffroyi zuluensis*.

During February 1951 several of these mites were collected on the same host from the Noodsberg Caves near Wartburg, Natal. They belonged to the species *Alabidocarpus nasicolus* which is already known, and to a new species of *Labidocarpus*, *L. natalensis*, which is related to the European *L. rollinati* Trouessart.

The South African representatives of these two genera also agree with their European relatives in being restricted to quite separate habitats. *Alabidocarpus nasicolus*, like the European *A. megalonyx*, is found only on the vibrissae which occur in the neighbourhood of the nose-leaf of the host, while *Labidocarpus natalensis* n.sp., like *L. rollinati*, lives in large numbers in the body fur.

A good series of another new species of *Alabidocarpus* closely related to *A. nasicolus* was taken from the bat *Myotis tricolor* in November 1950, and the numerous stages represented throw some light on the peculiar manner of reproduction in these highly specialized mites.

Alabidocarpus calcaratus n.sp. (Figs. 1, 2)

Types. Ten mature females, four males, four copulatory nymphs and eight immature forms at various stages, from *Myotis tricolor*, Town Bush Cave, Pietermaritzburg, November 1950.

Female (Fig. 1*a*). There is considerable heteromorphism of the sexes which is hardly less marked than in the genus *Labidocarpus*.

Apart from the marked difference of size, the fourth pair of legs of the female differs from those of the male in the epimera, which bear an incrassate, lanceolate, spur-like spine on their distal margin (Figs. 1*a*, 2*a*). In males and immature forms these are replaced by a pair of long, setiform spines occupying the same position. The female of this species resembles in general that of *Alabidocarpus nasicolus* (Lawr.) and has the same number of body annulations, namely, approximately 60. The characters which separate the two forms are enumerated on p. 139.

Male. The chief differences between the male and the female are that the male is smaller and the modified posterior end of its body is strongly chitinized and spined, as that of the male of *A. megalonyx*. This modification consists of two large laminae of thin, transparent chitin, each of which is armed near the distal margin with three large and conspicuous spines (Figs. 1*b*, 2*b*). The flap-like laminae are again divided and the spines appear to take their origin from between the two subdivisions. The laminae can be widely opened and function as a clasping apparatus, which closes against the sides of the posterior end of the abdomen of the copulatory female. The fixation of the sexes *in copula* is further effected by two genital adhesive disks, which are clearly seen through the transparent chitin. They are large and oval, and are situated near the hinge of each of the flap-like laminae, and are thus in close contact with the posterior end of the copulatory female. Trouessart (1895) did not describe adhesive disks in the male of *A. megalonyx*, the type of the genus *Alabidocarpus* (see Lawrence, 1948), but did attribute them to *Labidocarpus rollinati*. There is, however, no doubt that they are present in the males of both the South African species *Alabidocarpus calcarata* and *A. nasicolus* (Figs. 1, 2 and 4*d*), as well as in *Labidocarpus natalensis* (Figs. 3*a*, 4*a*). It seems very probable that they are also present in *Alabidocarpus megalonyx*.

The male further differs from the mature female in the following secondary characters: the main lateral body seta is shorter, while that of the tarsus of leg IV is rather longer than in the female; the claw of leg IV is relatively longer, exceeding the remainder of the leg; in the female it is definitely shorter. The number of body annulations is very constant, affording a reliable means of distinguishing not only the sexes but also closely related species. In the four male specimens they varied between 39 and 41.

The copulatory or apodous female. Three of the four specimens of mature males were taken *in copula* with this form of female. It is distinguished by the atrophy of the mouthparts and legs, the latter being

represented by four pairs of small round papillae, which are similar in shape, but the two posterior ones are surmounted by three spines, the anterior ones by apparently only two (Fig. 1*b*). The mouthparts can hardly be said to exist. They are represented by what appears to be a small fissure between the main portion and a small chitinized structure below it, which resembles a vertebrate jaw (Fig. 1*b*). The 'head' has no definable structure, but is weakly chitinized, the jaw-like portion being rather more so. In two of the apodous females the structures of the next stage developing within the body were very clearly defined. This stage occupied almost the whole volume of the body and resembled the mature or pre-mature form of female in having the two posterior pairs of legs fully developed, with long tarsal claws and tarsal spurs (two on the third and three on the fourth tarsus). In the more advanced stages the large spines of these legs could be clearly seen. In one of the apodous females the form of the developing next stage, although it was present in outline, was without definite structure.

So many different forms were represented in the material that they are best tabulated as follows:

Females	Males
(1) Limbless copulatory female, the legs represented by spined papillae; mouthparts absent or rudimentary; body containing the fully formed, but unchitinized 4-legged female of the next stage, which resembles the mature female in having strong spines on the fourth pair of legs. Length of body 446 μ.	—
(2) A 3-legged, fully chitinized form (the fourth pair of legs absent) with 55–60 body annulations; as in the copulatory female the whole of the body cavity usually occupied by the next developing stage, which is 4-legged, the fourth pair of legs having no long spines. Length of body 380 μ.	A 3-legged fully chitinized form (the fourth pair of legs absent); posterior third of abdomen smooth and without annulations; body with 28–29 annulations. Length of body 270 μ.
(3) A 4-legged fully chitinized form with 60 annulations; the fourth pair of legs without long spines on the tarsal and pretarsal segments, but with the usual spurs at the base of the claw on legs III and IV; the epimera of legs IV with a setiform spine as in the male, but shorter; the whole of the body cavity occupied by the next developing stage, which is 4-legged and has fully developed long and strong spines on legs IV, as in the adult female. Length of body 412 μ.	—
(4) A large, mature, fully chitinized female with 60 annulations; legs III and IV with long and strong spines; a pair of thickset, lanceolate spines on the epimera between legs IV (Fig. 2*a*); usually with a small hexapod larva in the body cavity (Fig. 1*a*). Length of body 730 μ.	Mature forms with 40 annulations; posterior end of abdomen much modified with laminate, chitinous claspers provided with three strong spines; posterior legs as in adult female, but a pair of long setiform spines on the epimera between legs IV. Length of body 380 μ.

The developmental cycle thus appears to be rather complicated, but the various stages described above can probably be related to each other in the following sequence:

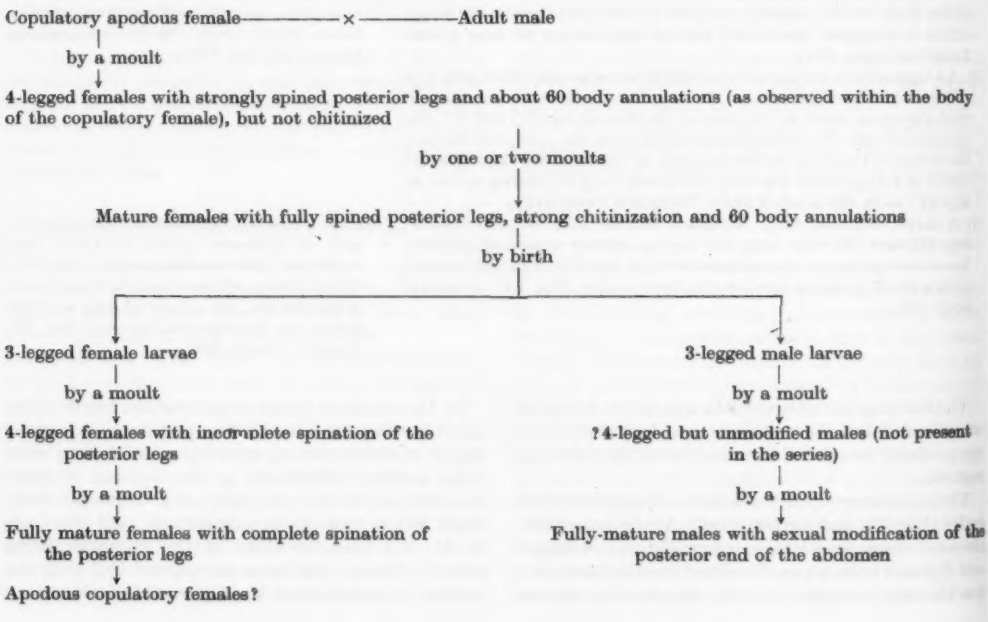
The copulatory apodous females copulate with the males (Fig. 1*b*) and are fertilized. At the same time, the next stage, which is, in every instance, 4-legged and appears to be an unchitinized female, because it has the large number of body annulations characteristic of the adult form, ± 60, can be seen occupying the whole body cavity. This next stage already has the strong spines on legs IV, which are present in the adult female. These females appear after a moult of the apodous females and are then either fully mature or become so after another moult, when they acquire fully chitinized structures. They have presumably been fertilized while still in the apodous stage. The next phase seems to be a reproductive one, and the adult females, being provided with sperms carried over from the previous stage, produce hexapod larvae. As the author (1938) has already reported, many adult females of *A. nasicolus* contain a single 3-legged larva, which occupies a comparatively small part of the body with the head always directed posteriorly. The larvae of *A. calcaratus* are at various stages of chitinization from transparent to quite dark and heavily chitinized forms. The size of the larva makes it probable that its birth or emergence must inevitably cause the death of the female. A larva *in situ*, showing its size and position in relation to the female, is shown in Fig. 1*a*. The very similar state of affairs in *A. nasicolus* is figured by the author (1938, Fig. 3).

In the material under consideration, eight of ten adult females contained a hexapod larva at various stages of chitinization, showing even at this early stage marked differences in the number of body annulations, which are quite easy to count. Thus three larvae had 55–61 annulations, and five only 28–31, the posterior third of the abdomen being smooth. These differences correspond well with the number of annulations in adult males and females.

The mature female then probably produces 3-legged forms of both sexes, while males are derived from her by parturition.

The hexapod larvae, after birth, probably develop into 4-legged nymphs (stage 3) by a moult, and these by another moult into fully mature and heteromorphic males and females. In these mites it would seem that the males for various reasons are able to copulate only with the smaller apodous and unchitinized females and that the larger mature female can only derive spermatozoa from the male via the apodous stage. The mature female produces only hexapod larvae of both sexes by birth and these develop by a series of moults into adult males and females. The apodous copulatory female, on the other hand, develops into a 4-legged mature female by a series of moults. We do not yet know how the apodous female, which is a stage of considerable size, and is as big as the adult male, is itself derived. The apodous female appears only *in copula* with the male, and there are no previous stages leading up to it. There is nothing in the bodies of the female forms suggesting the appearance of an egg, although the outline of the contents of the bodies of a few of the females of *A. nasicolus* gives an impression that in some instances the apodous female may be derived from this stage, i.e. that some of the mature females revert to the apodous form and that the life cycle begins afresh.

The conjectural sequence of development can be summarized in the following manner:


Copulatory apodous female × Adult male
by a moult
4-legged females with strongly spined posterior legs and about 60 body annulations (as observed within the body of the copulatory female), but not chitinized
by one or two moults
Mature females with fully spined posterior legs, strong chitinization and 60 body annulations
by birth
3-legged female larvae
by a moult
4-legged females with incomplete spination of the posterior legs
by a moult
Fully mature females with complete spination of the posterior legs
Apodous copulatory females?
3-legged male larvae
by a moult
?4-legged but unmodified males (not present in the series)
by a moult
Fully-mature males with sexual modification of the posterior end of the abdomen

Alabidocarpus nasicolus (Lawr.)

Labidocarpus nasicolus Lawrence (1938).

Alabidocarpus nasicolus (Lawrence) (1948).

Three females and one male were taken from the type host, *Rhinolophus geoffroyi zuluensis*, at the type locality, Noodsberg Caves, Wartburg, Natal, in February 1951.

Only the female of this species has been described, the following being a description of the male.

Male. The chitinous hood covering the dorsum of the head anteriorly above the anterior legs with an angular backwardly projecting lobe, as in the female; just posterior to this hood two small spines near the dorsal margin; main lateral body spine, just above the insertion of leg III, long and very stout in its basal third, the remainder slender and tapering; just below the insertion of the main spine a very short and inconspicuous spine.

Legs. Epimera of legs IV with a pair of long, setose spines near their distal margin, reaching to the base of the tarsal spur, thick at their bases, but finely tapering; leg IV distinctly longer than leg III, but not as thickset; claw of leg IV much curved in its apical third, longer than the remainder of the leg; apical seta of tarsus IV exceeding the claw.

Posterior modified region of abdomen as in Fig. 4*d*, differing from the male of *Alabidocarpus calcaratus* in its general shape and in having the middle spine of the laminated claspers twice or a little more the length of the remaining two; two

large, almost round adhesive disks clearly visible. In *A. calcaratus* the male is only about half the length of the female, while in *A. nasicolus* it is a little more than two-thirds. Body with 40 annulations.

The manner of feeding in Alabidocarpus

The manner of feeding and the food substances taken by these highly specialized mites has remained

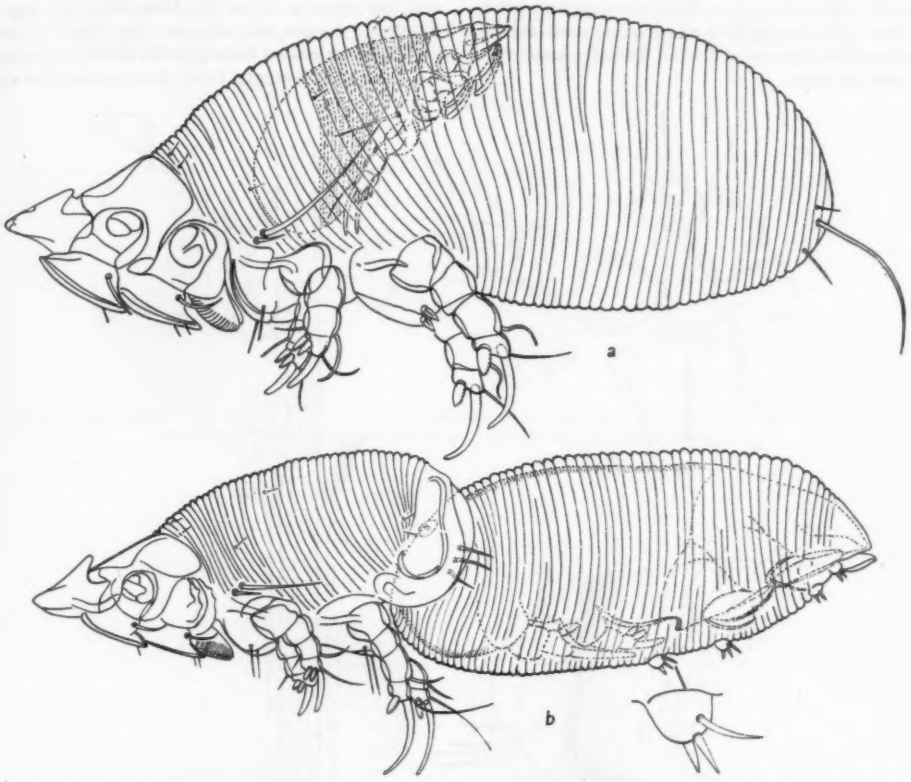


Fig. 1. *Alabidocarpus calcaratus* n.sp. *a*, mature female with hexapod larva seen through the body wall; *b*, male attached to a copulatory female, the latter with the next 4-legged stage seen through the body wall.

Dimensions. Length of body 465 μ.

The females of *A. nasicolus* and *A. calcaratus* can be easily distinguished by the following characters:

A. calcaratus	*A. nasicolus*
Epimera of legs IV with a pair of short, incrassate, lanceolate spines resembling the tarsal spurs (Fig. 2*a*)	Epimera of legs IV with a pair of long setiform spines
Anterior dorsal hood rounded posteriorly (Fig. 1*a*)	Anterior dorsal hood with a sharp, angular projection posteriorly
Main lateral body spine above leg III with a conspicuous short, stout spine just below it (Fig. 1*a*)	Main spine with or without a very small and inconspicuous spine below it
A short spine between the main lateral spine and the dorsal margin of the body (Fig. 1*a*)	No such spine
A small spine at the level of the posterior margin of epimera IV near the dorsal margin of the body (Fig. 1*a*)	No such spine
A minute spine just above the main flagelliform seta at the posterior end of the body (Fig. 1*a*)	No such spine

a matter for conjecture. A specimen of *A. calcaratus* taken from the host and mounted at once while still attached to a hair (Fig. 2*c*) suggests the manner in

which these mites may feed. The specimen is a 3-legged male, which evidently uses the flattened, shovel-like head to strip away the outer layers of the hair as it passes downward toward the follicle. The vibrissae of the nose-leaf of the host seem to be clotted with soft, fatty tissues near their roots and it may be that the mite is able to extract some nourishment from these substances and from the soft parts of the hair near its base.

Both sexes closely resemble the European species *L. rollinati* Trouessart (1895).

Male (Fig. 3*a*). Size small; the posterior modified genital apparatus with two adhesive disks and as strongly chitinized as the anterior part of the head and far more so than in *Alabidocarpus*; legs III distinctly longer and stouter than legs IV, which are often concealed between the third pair; sides of the body with a single, fairly short spine, just above

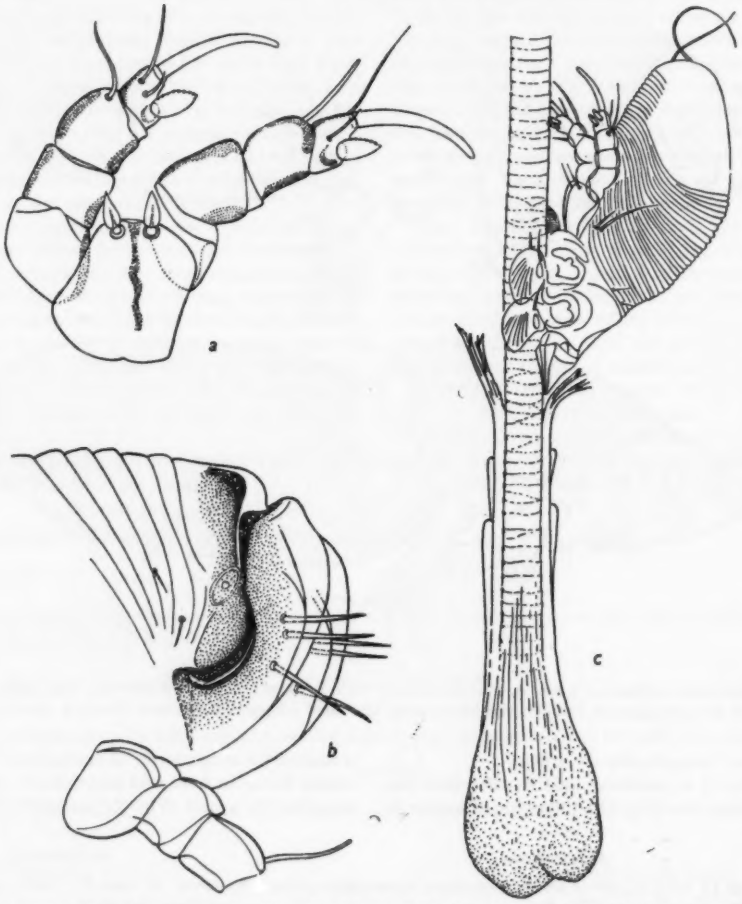


Fig. 2. *Alabidocarpus calcaratus* n.sp. *a*, fourth pair of legs of the female viewed from below; *b*, posterior apex of abdomen of a mature male to show the two claspers of the copulatory apparatus; *c*, a young 3-legged male attached to a hair of the host near the follicle.

Labidocarpus natalensis n.sp. (Figs. 3, 4)

Types. Seven males attached to copulatory females; eight mature females, from *Rhinolophus geoffroyi zuluensis*, Noodsberg Caves, Wartburg, Natal, February 1951.

the insertion of leg III, stout at its origin, but tapering finely; body otherwise unspined, except for a pair of moderate setae on the dorsal margin just behind the posterior termination of the cephalic hood, and one pair of long and one pair of short flagella rising from the chitinized portion of the

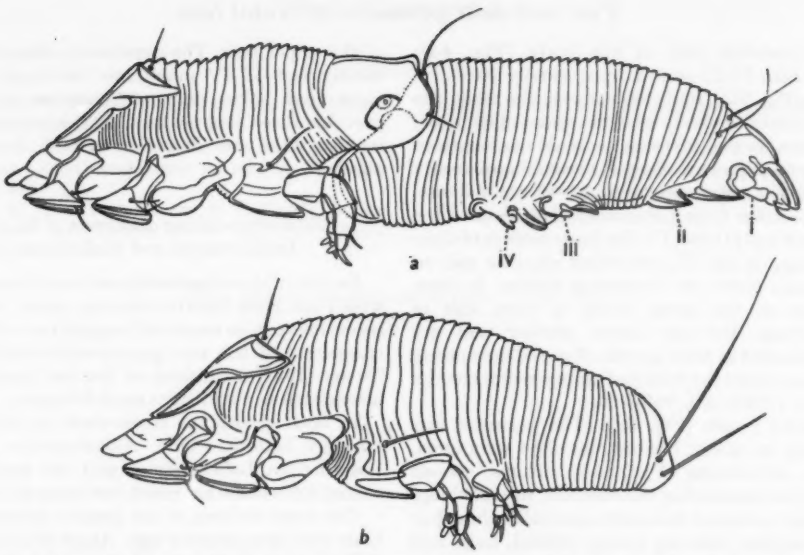


Fig. 3. *Labidocarpus natalensis* n.sp. *a*, the male attached to the copulatory female, the rudimentary legs of the latter indicated by roman numerals; *b*, mature female.

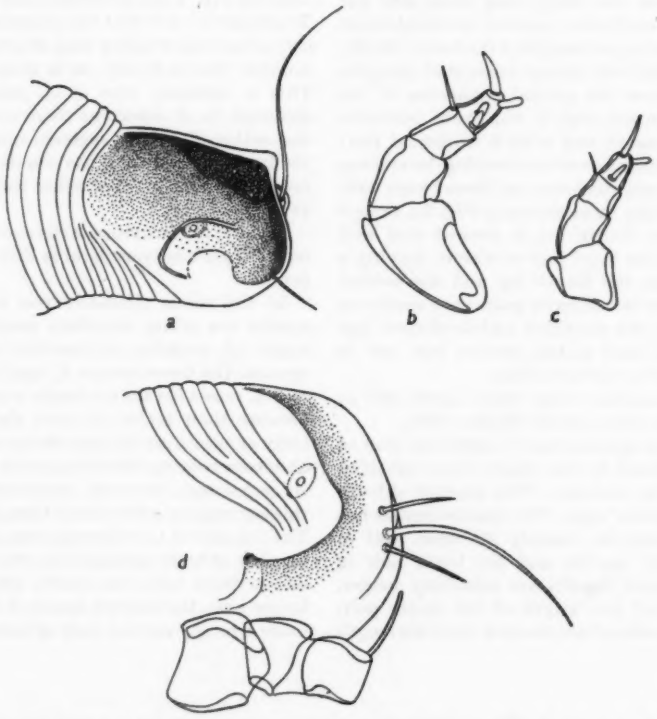


Fig. 4. *a*, posterior apex of the abdomen of a mature male, *Labidocarpus natalensis*, n.sp.; *b*, leg III of the same; *c*, leg IV of the same; *d*, posterior apex of the abdomen of a mature male, *Alabidocarpus nasicolus* (Lawr.). Only the left-hand clasper is shown.

truncate posterior end of the body (Fig. 4*a*); abdomen with 18–21 annulations, usually 20.

Female (Fig. 3*b*). Body spined as in the male; the posterior end of the body with two pairs of flagellae, the lower being two-fifths to one-half the length of the upper one; epimera of legs III and IV apparently without spines; abdomen with 37–43 annulations. Both sexes differ from *Labidocarpus rollinati* in the spination of legs III and IV, the inner surface of their tarsi having, on leg III, two blunt papillae and on leg IV, one; these are somewhat similar in their appearance to the tarsal spurs of these legs in *Alabidocarpus*, but are much smaller and are bluntly rounded at their apices. None of the mature females contained the hexapod embryos reported by Trouessart (1895) in *L. rollinati*.

Copulatory female (Fig. 3*a*). The posterior legs represented by short, bilobed, bud-like structures, with faint indications of joint-like sutures; second (anterior) leg resembling the anterior, modified legs of the adult males and females in general outline, but far less complex; first leg clearly defined, large and with three segments, the terminal segment with a short spine; head region better developed and more strongly chitinized than in the copulatory female *Alabidocarpus*, with two fairly long setae near the dorsal margin on each side; number of annulations (counted along the upper margin of the body) 33–36; posterior end of the body lightly excavated along its upper margin where the genital apparatus of the male comes in contact with it, the lower posterior portion being rounded and with a thickened rim; none of the copulatory females containing developing stages with a distinct structure; all these forms with rudimentary legs like those shown in Fig. 3*a*, except one, in which the fourth leg is present and well developed, while the third pair is absent, leaving a large gap between the fourth leg and the second (anterior) pair; the two anterior pairs very similar in their structure to the modified paddle-shaped legs of the adults; a head shield present but not so strongly chitinized as in the adults.

Dimensions. Length of the body: male, 267 μ; copulatory female, 284 μ; adult female, 330 μ.

The male of this species closely resembles that of *Labidocarpus rollinati* in the shape of the modified posterior end of the abdomen. The number of body annulations is also the same. The mature female has more body annulations, namely 40, than that of *L. rollinati* which has 30, and the lower pair of posterior abdominal flagella are relatively longer, namely, about half the length of the upper pair, while those of *L. rollinati* are about a third the length of the upper pair. The copulatory female differs in having legs II–IV much less developed and unsegmented. The pair of conspicuous rounded protuberances (mamelons), on the posterior upper margin of the abdomen of *L. rollinati*, are markedly absent.

The distinguishing characters of the genera Labidocarpus *and* Alabidocarpus

In view of the considerable amount of new material which has been described in this paper, certain observations can be made with regard to the distinctive characters of the two genera with which it deals. To my (1948) definitions of the two genera certain alterations and additions must be made.

In both genera the sexes could be described as strongly heteromorphic, *Alabidocarpus* being not less so than *Labidocarpus*, and the nature of the sexual differences are much the same in both.

The most striking of the generic differences have to do with the posterior legs. Apart from the marked differences in the tarsi and claws of the two genera, leg III in *Labidocarpus* is decidedly longer and stouter than leg IV, while in *Alabidocarpus*, leg IV is longer than leg III. I am, however, now inclined to accept Trouessart's view that the posterior legs of *Alabidocarpus* have four rather than five segments, the same number, that is to say, as in those of *Labidocarpus*. This is certainly true of *A. calcaratus* (Fig. 2*a*), although in *A. nasicolus* there seems to be a small and rather ill-defined segment intercalated between the basal segment and the one distal to it. There is, however, some doubt whether this could be counted as a true segment.

Finally, the structure of the copulatory or apodous female exhibits considerable differences in the two genera.

In the genus *Alabidocarpus* the two European species are either definitely smaller (*A. minor*) or larger (*A. megalonyx*) than the two South African species. The three species *A. megalonyx*, *A. calcaratus* and *A. nasicolus* are evidently closely related forms, because their males all have the same number of body annulations (40) and the same type of modified abdomen bearing three long stout spines posteriorly. *A. megalonyx*, however, resembles *A. calcaratus* in this last respect more closely than it does *A. nasicolus*. The females of the three species also have the same number of body annulations (60), but *A. megalonyx* differs from both the South African forms in its larger size, the shorter spurs of tarsus III and the more strongly curved claw of tarsus IV.

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ON THE MORPHOLOGY AND LIFE HISTORY OF A NEW MAMMALIAN BLOOD-FLUKE—*ORNITHOBILHARZIA DATTAI* N.SP. (PRELIMINARY REPORT)

By S. C. DUTT, M.Sc. and H. D. SRIVASTAVA, M.Sc., D.Sc.
Indian Veterinary Research Institute, Izatnagar, India

(With 11 Figures in the Text)

Of the fifteen valid species of mammalian blood-flukes so far known, six have been originally described from India. Montgomery (1906) described *Schistosoma indicum*, *S. spindalis* and *Ornithobilharzia bomfordi* (Montgomery, 1906) Price, 1929. Datta (1932) found that nasal granuloma of cattle is due to a new schistosome, which was subsequently obtained and described as *Schistosoma nasalis* by Rao (1933). Rao & Ayyar (1933) obtained a new schistosome from pigs and described it as *S. suis*. Mudaliar & Ramanujachari (1945) described *S. nairi* n.sp., from an elephant, which was transferred to the genus *Ornithobilharzia* by Bhalerao (1947). Another new species of blood-fluke has now been found in Indian buffaloes and cattle. While studying the larval trematode fauna of the local aquatic snails, large numbers of a new schistosome cercaria were obtained from nine out of three hundred specimens of *Limnaea luteola* examined during November 1950. When the cercariae were administered to two guinea-pigs, a buffalo-calf and a bull-calf, they developed into an interesting blood-fluke referable to the genus *Ornithobilharzia* Odhner, 1912. Subsequently, three out of thirteen buffaloes examined post-mortem were found to be naturally infected with the same parasite. The morphology of the adult and of its cercaria is briefly described in this paper.

MORPHOLOGY OF THE ADULT

The material for the study of the adult flukes consisted of 229 males obtained from the portal veins of two guinea-pigs infected experimentally; 664 males, twenty-eight females and seven pairs *in copula* from the portal and mesenteric veins of a buffalo-calf infected experimentally; and seventy-eight males, eleven females and one pair *in copula* from three buffaloes infected naturally. The specimens were fixed in Gilson's mixture, Bouin's fluid and 5% formalin.

Male (Fig. 1)

Body 3·042–7·6 mm. long and 0·114–0·243 mm. in maximum width in the region of the testes. Pre-acetabular portion of the body about one-tenth of the whole length, flat, having two distinct free edges. Oral sucker subterminal, spinose, 0·114–0·243 mm. long and 0·085–0·157 mm. wide. Ventral sucker pedunculate and spinose, 0·135–0·243 mm. in diameter. Distance between the oral and ventral suckers 0·3–0·657 mm. Post-acetabular portion of the body long and flattened, with the lateral edges infolded ventrally, forming a well-developed gynaecophoric canal. Cuticle atuberculate and spiny. Oesophagus with a posterior swelling. Paired intestine long, posteriorly joined by transverse commissures before finally uniting to form the common caecum which is 0·4–0·25 of the length of the paired intestine. Post-caecal portion of the body 0·087–0·166 mm. long. Testes 37–66 in number and 0·06–0·067 by 0·033–0·043 mm. in size, arranged dorsally in a single row. Seminal vesicle 0·067 × 0·046 mm. long, elliptical, situated a little distance in front of the most anterior testis. Genital pore median, opening just posterior to the acetabulum.

Female (Figs. 2, 4 and 5)

Filiform, 3·91–7·51 mm. long, widest in the region of the ovary, where its width is 0·067–0·107 mm. Width between the oral and ventral suckers 0·027–0·04 mm. Width at the region of the caecal termination 0·02–0·035 mm. Cuticle spiny, especially on the oral and ventral suckers. Oral sucker subterminal, measuring 0·043–0·073 by 0·027–0·037 mm. Ventral sucker distinctly pedunculate, 0·027–0·037 mm. in diameter. Oesophagus with a posterior swelling. Paired intestine 2·23–4 mm. long, longer than the common caecum. Common caecum 1·57–3·07 mm. long. Post-caecal portion of the body 0·057–0·143 mm. long. Ovary 0·2–0·266 mm. in length and 0·043–0·09 mm. in maximum width, situated in the posterior half of the body, cephalad of the caecal union. It consists of a broader, compact, elliptical posterior portion and a more slender, slightly coiled, tubular, anterior portion. The oviduct originates from the posterior end of the ovary and, after a short coiled course, proceeds forwards. Uterus long, having

a posterior elliptical swelling containing a single egg, immediately posterior to which the oviduct and the vitelline duct unite. Genital pore situated immediately behind the acetabulum. The vitellaria consist of transversely elongated follicles situated on each side of the common caecum, extending from the caecal union to the posterior end of the body up to a little distance cephalad of the caecal termination. Egg asymmetrically oval often with one side slightly flattened, with a terminal spine the distal end of which is slightly bent. The shape of the uterine eggs varies considerably. Some have typically the shape of the eggs of *S. bovis*; one side of others is much flattened, while some are much shorter and broader. Uterine eggs 0·073–0·11 mm. long, including the spine, and 0·023–0·04 mm. broad, the spine being 0·007–0·017 mm. long. Voided eggs 0·12–0·137 mm. long with a maximum width of 0·043 mm.

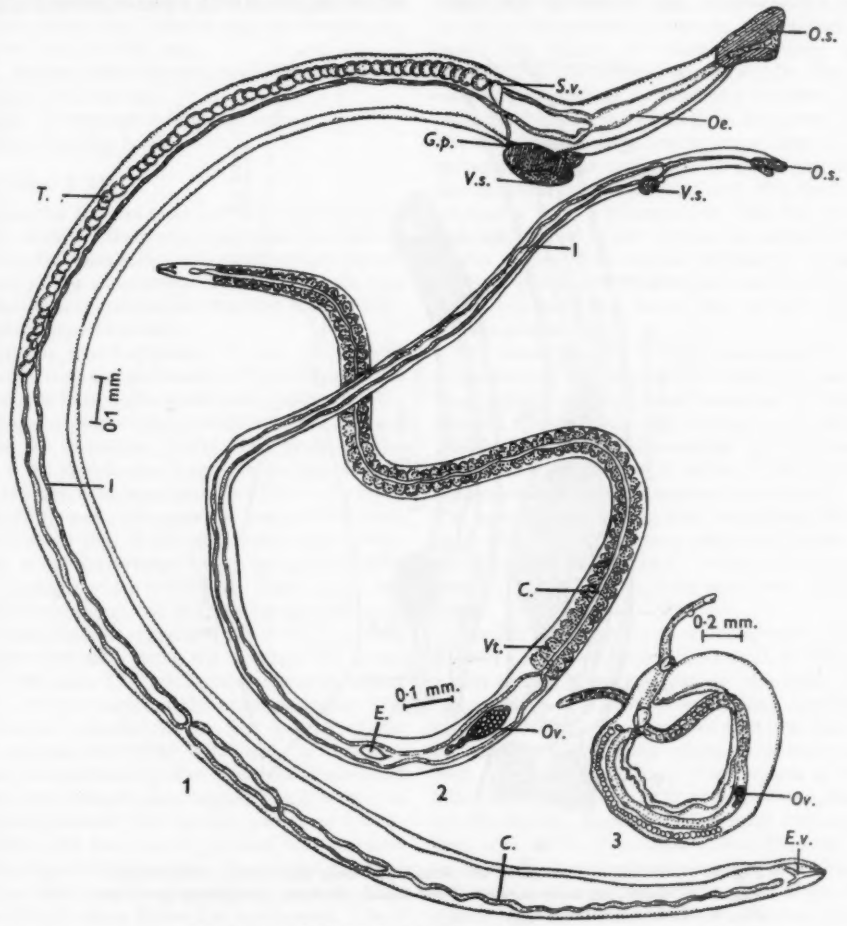

Fig. 1. *Ornithobilharzia dattai*, male.
Fig. 2. *O. dattai*, female.
Fig. 3. *O. dattai*, male and female *in copula*.

The blood-fluke described in this paper is most closely related to the genus *Ornithobilharzia* Odhner, 1912. Members of this genus occur as adults in the circulatory systems of birds and mammals. The ten species known to parasitize birds are: *O. intermedia* Odhner, 1912, *O. canaliculata* (Rudolphi, 1819) Odhner, 1912, *O. kowalewskii* (Paronia & Areola, 1896) Odhner, 1912, *O. odhneri* Faust, 1924, *O. pricei* Wetzel, 1930, *Ornithobilharzia* sp. Gogate, 1934,

O. lari McLeod, 1937, *O. aviani* McLeod, 1940,* *O. filamentous* McLeod, 1940* and *O. emberizae* Yamaguti, 1941.* The species which occur in mammals are: *O. bomfordi* (Montgomery, 1906) Price, 1929, *O. turkestanicum* (Skrjabin, 1913) Price, 1929, *O. turkestanicum* var. *tuberculata* Bhalerao, 1932, *O. nairi* (Mudaliar & Ramanujachari, 1945) Bhalerao, 1947, and *O. dattai* n.sp. The new species can be distinguished from the various known species on the basis of several characters such as the nature of cuticle, relative sizes of the male and the number of testes and the proportionate lengths of the paired intestine and the common caeca, etc. But the most important character which differentiates it from all the avian and mammalian species is the position of the ovary. The ovary of *O. dattai* is situated in the posterior half of the body, while in all the other species it is pre-equatorial in position. The species is assigned to the genus *Ornithobilharzia* Odhner, 1912, tentatively, with its generic diagnosis modified to include also forms with a post-equatorial ovary.

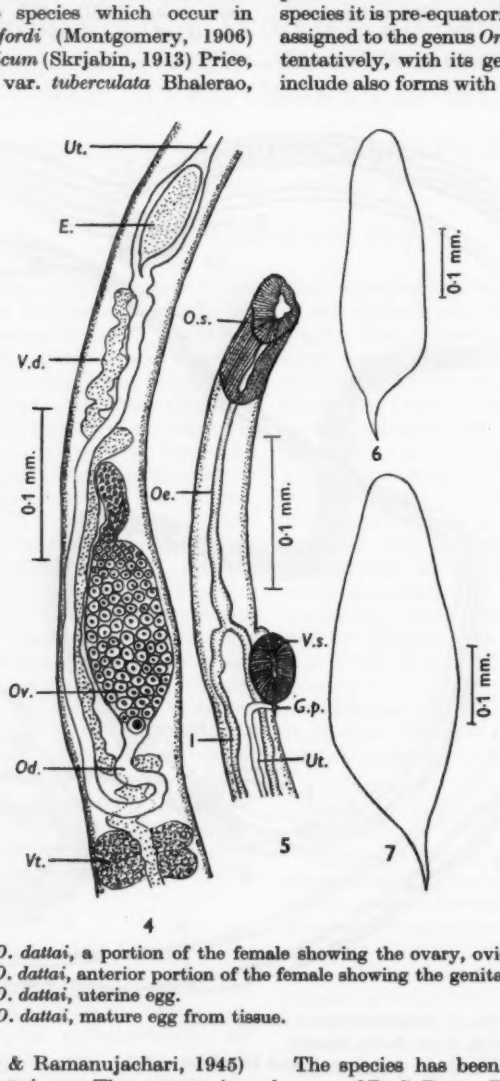

Fig. 4. *O. dattai*, a portion of the female showing the ovary, oviduct, etc.
Fig. 5. *O. dattai*, anterior portion of the female showing the genital pore, etc.
Fig. 6. *O. dattai*, uterine egg.
Fig. 7. *O. dattai*, mature egg from tissue.

The species has been named *O. dattai* n.sp., in honour of Dr S. Datta, D.Sc., M.R.C.V.S., Director, I.V.R.I., Izatnagar, whose extensive researches on the etiology of several obscure helminthic diseases have been a source of inspiration to the authors in their studies in helminthology.

Specific diagnosis of Ornithobilharzia dattai *n.sp.*

Male. Length 3·042–7·6 mm. Maximum width 0·114–0·243 mm. Cuticle atuberculate. Testes 37–66 in number.

* The full descriptions of these species have not so far been available to the authors.

Female. Length 3·91–7·51 mm. Maximum width 0·067–0·107 mm. Paired intestine longer than common caecum, being 2·23–4 mm. long. Common caecum 1·57–3·07 mm. long. Ovary post-equatorial. Uterus containing a single egg. Egg asymmetrically oval, with a terminal spine. Uterine eggs 0·073–0·11 × 0·023–0·04 mm. Mature eggs in tissues and faeces 0·12–0·137 × 0·043 mm.

Hosts. Buffalo (*Bos bubalus*); cattle (*Bos indicus*); guinea-pig (experimental).

Location. Portal and mesenteric veins.

Locality. Bareilly, India.

Cercaria (Figs. 8–11)

The material for the study of the cercaria of *O. dattai* was obtained from nine specimens of *Limnaea luteola* infected naturally and twenty-eight specimens bred in the laboratory. The latter snails had been infected with miracidia obtained from a bull-calf infected experimentally.

The cercaria, which appears to be new, is apharyngeal, brevifurcous and non-oculate. Eighty specimens of the cercaria from eight snails were measured after killing them by gentle heat according to the method employed by Soparkar (1921). The body of the cercaria is 0·135–0·2 mm. long, the average length being 0·168 mm. The maximum width of the body is 0·044–0·065 mm., the average being 0·058 mm. The tail-stem is 0·21–0·316 mm. long and 0·026–0·039 mm. wide, the average length being 0·258 mm. and the average width 0·032 mm. The furcae are 0·071–0·116 mm. long, the average being 0·097 mm. The pyriform anterior organ is 0·052–0·077 mm. long and 0·032–0·035 mm. wide, the average size being 0·055 × 0·032 mm. The head-gland occupies about four-fifths of the length of the anterior organ. The ventral sucker, situated in the last quarter of the body, is spinose and 0·016–0·023 mm. in diameter and has a Y-shaped cavity. The mouth is subterminal and leads to a narrow oesophagus, which ends in a conspicuous caecum. The caecum measures 0·006–0·01 × 0·01–0·013 mm. and is bilobed and situated behind the equator of the body. It contains granules of various sizes, which are stained brightly with neutral red and show Brownian movement. There are five pairs of penetration glands, namely, two anterior pairs, which are coarsely granular and three posterior pairs, which are finely granular. The anterior tip of each of the five pairs of ducts is capped by a hollow piercing spine. The nervous system is represented by two thick, hemispherical masses, which occupy the second quarter of the body. The two masses are connected by a thin median commissure lying dorsal to ducts of the penetration glands. The excretory system is shown in Fig. 8. The flame-cell formula is $2[2+1(+1)]=6(+2)$. The most anterior body flame-cell is situated a little distance behind the anterior border of the nerve mass. The second anterior body flame-cell is situated at about the level of the anterior margin of the second penetration gland; usually it is directed medially, but occasionally its direction may be the reverse. The main lateral collecting tube has two ciliated areas. The posterior body flame-cell is situated lateral to the genital rudiments, sometimes further back. The island of Cort is present at the beginning of the caudal excretory tube. The pair of caudal flame-cells is situated at a distance of about one-eighth of the tail-stem from the proximal end. The furcal excretory tubes originate from the caudal excretory tube at a distance of about one-tenth of the tail-stem from the distal end. The flask-shaped dilatation (Fig. 10) connected with the excretory opening is situated well within the margin of the tip of the ramus. The genital rudiments consist of a group of germ cells behind the ventral sucker. The body, tail-stem and furcae are covered with prominent spines.

The cercariae can be easily seen with the naked eye when they are observed in water in a glass vessel. They remain uniformly distributed in the water and float tail upwards with the furcae spread horizontally. The movements of the cercariae in water and on a substratum are similar to those of the cercariae of *Schistosoma spindalis*, described by Soparkar (1921). The cercariae develop in thin-walled tubular sporocysts (Fig. 11), which may easily be broken during the dissection of the liver. A few entire sporocysts were 0·714–1·571 mm. long and 0·057–0·157 mm. wide.

The life history of none of the species of *Ornithobilharzia* is known except that of *O. turkestanicum*, which has been worked out by MacHattie (1936). The cercaria of *O. turkestanicum* has, however, not been described completely, so that the description of the cercaria of *O. dattai* given above is perhaps the first complete description of a cercaria of *Ornithobilharzia*. The cercaria of *O. turkestanicum*, described by MacHattie, appears to be quite different from that of *O. dattai*. The latter differs from the former in its size and proportionate lengths of the body and tail-stem and in possessing five pairs of penetration glands intead of four pairs present in the cercaria of *O. turkestanicum*. Further, the penetration glands of the cercaria of *O. turkestanicum* contain finely granular protoplasm, while the two anterior pairs of penetration glands of the new cercaria are coarsely granular and the three posterior pairs finely granular. The penetration glands of the cercaria of *O. dattai* resemble those of the cercariae of *S. haematobium*, *S. mansoni*, *S. bovis*, *S. spindalis*, *S. nasalis* and *S. indicum* in their number and the nature of their granulation. The cercaria of *S. japonicum* also has the same number of glands, but their granulation is not the same. The excretory system of the cercaria of *O. turkestanicum* has not been worked out.

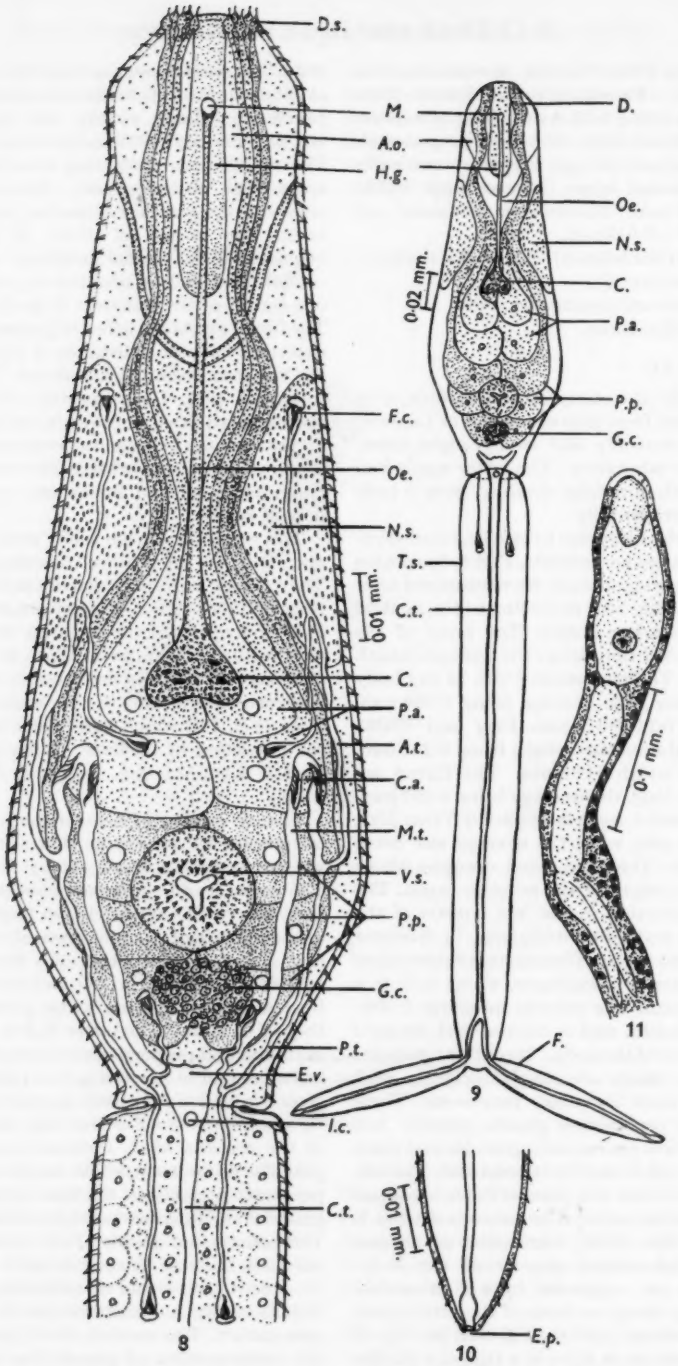

Fig. 8. Cercaria of *O. dattai*, the body and a portion of the tail, showing various structures.
Fig. 9. Cercaria of *O. dattai*, showing the body, tail-stem and furci.
Fig. 10. Cercaria of *O. dattai*, showing the excretory pore at the tip of the furcal ramus.
Fig. 11. A portion of a sporocyst showing a mature cercaria.

The cercaria of *O. dattai* can be distinguished easily from the cercariae of the *Schistosoma* species. Apart from differences in size and relative proportions of body parts, it differs from the cercariae of *S. haematobium* and *S. mansoni* (Gordon, Davey & Peatson, 1934), and those of *S. spindalis* (Soparkar, 1921) and *S. indicum* (Srivastava & Dutt, 1951) in possessing four instead of five pairs of flame-cells. The cercaria of *Ornithobilharzia dattai* differs from the cercaria of *Schistosoma nasalis* (Sewell, 1922; Rao, 1934) in the size and shape of the caecum, and in the relative lengths of the body and tail-stem. The cercaria of *S. japonicum* (Faust & Meleny, 1924) differs from the cercaria of *Ornithobilharzia dattai* in size, relative lengths of the body and tail-stem and in the nature of granulation of the penetration glands. The cercaria of *Schistosoma bovis* (Porter, 1938) differs from the cercaria of *Ornithobilharzia dattai* in its size and in the position of the anterior flame-cells.

Further work is in progress on the details of the life history and biology of the parasite and on its host-parasite relationship.

The authors are greatly indebted to Dr S. Datta, D.Sc., M.R.C.V.S., Director of this Institute, for his keen interest and encouragement in these studies. They are also grateful to Dr H. N. Ray, M.Sc., Ph.D., Officer-in-Charge of the Section of Parasitology, for providing the requisite facilities.

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Key to lettering on figures

A.o. anterior organ; *A.t.* anterior collecting tubule; *C.* caecum; *C.a.* ciliated area; *C.t.* caudal excretory tube; *D.* duct of the penetration gland; *D.s.* duct spine; *E.* egg; *E.p.* excretory pore; *E.v.* excretory vesicle; *F.* furca; *F.c.* flame-cell; *G.c.* germ cells; *G.p.* genital pore; *H.g.* head gland; *I.* intestine; *I.c.* island of Cort; *M.* mouth; *M.t.* main lateral collecting tube; *N.s.* nervous system; *Od.* oviduct; *Oe.* oesophagus; *O.s.* oral sucker; *Ov.* ovary; *P.a.* anterior penetration glands; *P.p.* posterior penetration glands; *P.t.* posterior collecting tubule; *S.v.* seminal vesicle; *T.* testes; *T.s.* tail-stem; *Ut.* uterus; *V.d.* vitelline duct; *V.s.* ventral sucker; *Vt.* vitellaria.

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IXODES FRONTALIS PANZER, 1795. A REVIEW OF THE SPECIES

By D. R. ARTHUR

Department of Zoology, King's College, University of London

(With 8 Figures in the Text)

The identification of *Ixodes frontalis* Panzer, 1795, from earlier descriptions presented great difficulty, more particularly as the species is rare. There are two specimens in the British Museum (Natural History), and the description by Schulze (1933) is from a single specimen in the Sofia Museum. The rarity of the species led Schulze to write that 'Die Art ist in Deutschland seit den Zeiten Panzers nicht mehr wiedergefunden worden'. Senevet (1937), who considers *I. frontalis* synonymous with *I. brunneus* Koch, 1844, wrote that the species occurred in St Genies-de-Malgoires in France, but this information was taken from Nuttall, Warburton, Cooper & Robinson (1911).

A new description is therefore presented, which it is hoped will place the classification of the species on a more satisfactory footing. This description is based on one fully fed and one unfed female tick collected from two blackbirds at Silwood Park, Berks, and on a fully-fed female from a great tit (Ashburton, South Devon). The three specimens are similar and were examined before and after mounting. As in *I. brunneus* Koch (Cooley & Kohls, 1945) the denticle arrangement on the hypostome can be ascertained only after dissection and microscopic examination. Previous examinations of the hypostome *in situ* may possibly have led to inaccuracies.

Body. Oval, wider behind the middle. Length (unfed) 3 mm., width 1·5 mm. Scutum reaching back more than half the length of the body. Fully fed specimens measure 11 by 6 mm. with the greatest width behind the spiracle. Scutum and alloscutum with a large number of conspicuous white hairs.

Capitulum (Figs. 3, 6). Length 0·76 mm., width of basis 0·51 mm. (across cornua). Lateral profile corrugated. Posterior margin straight or a little concave. Cornua rounded apically, postero-laterally directed and prominent. Porose areas large with distinct boundaries, subtriangular in outline, interporose distance less than the greatest breadth of the porose areas. Palpi long, narrow, with outer profile lines nearly straight, inner edges convex. Articles 2:3::2·2:1·7; combined lengths of 2 and 3, 0·61 mm. Hairs very numerous and long, especially on lateral margins.

In ventral view, basis has a slightly convex posterior margin, with a slight constriction behind the auriculae. The latter are large, laterally directed protuberances terminating bluntly. Basis widest posteriorly, salient. Surface crazed and shining.

Hypostome (Fig. 1). Long, narrow, pointed. Denticles arranged 4/4, 3/3, then 2/2 to the base, with a median triangular unarmed area. Length about 0·47 mm.

Scutum (Fig. 2). Length 1·47–1·49 mm., width 1·05–1·07 mm., widest in front of the middle, rounded behind. Cervical grooves as valleys separating the lateral and median areas, extending to postero-lateral margins. Median area more heavily pigmented than the lateral area. Lateral carinae present as sharp ridges, which are steep-sided. Surface irregular, glossy. Punctations small, absent in cervical grooves. Hairs long and white. Scapula rounded, emargination distinct.

Legs. Long and large, surface faintly crazed. Hairs numerous and long on the ventral surface, shorter and less abundant on the dorsal surface. Depth of colour in living specimens varies from segment to segment and from leg to leg.

Tarsi (Figs. 4, 5). Long, with slight hump, Haller's organ on tarsus I distant from apex. Length of tarsus I, 0·78 mm.; metatarsus I, 0·46 mm. Length of tarsus IV 0·66 mm.; metatarsus IV, 0·54 mm.

Coxae (Fig. 8). All coxae mildly convex, punctate and glossy. Coxa I with external spur and an internal spur of about the same length. External spurs on all coxae moderate in length. Trochanters I to IV with small but distinct ventral spurs.

Spiracular plate (Fig. 7). Heavily pigmented, oval. Length 0·37 mm.; width 0·31 mm., with longer axis transverse, macula central. Goblets numerous, five rows on the dorsal side, two on the ventral side.

Genital aperture. Situated between third intercoxal space (unfed female).

Anus. Placed well back on the ventral surface posteriorly, anal grooves fused in semicircle in front of the anus, short, terminating in front of the posterior border.

Panzer's description, 'oblongus ater nudus capite antennis pedibusque testaceris', and his figures fail to convince me that he was figuring what is now called *I. frontalis*. The size, given alongside the figure, is not in agreement with that of the female of

this species. The host was *Parus major*. This is also true for *I. pari* of Leach (1813), collected from the same host. Berlese (1890) described *I. avisugus* from birds in Florence and Venice, but the absence of external spurs on the coxae, the form of the porose areas and the scutum do not agree with our data. The hypostome is not figured.

Sunol, California (Nuttall collection, N. 283) and having examined this material, I agree with Cooley & Kohls.

In the Nuttall collection (British Museum) there are only two specimens of *Ixodes frontalis* from England, one from an owl (cat. no. N. 224) and the other from a wagtail (cat. no. 2546). Both were originally

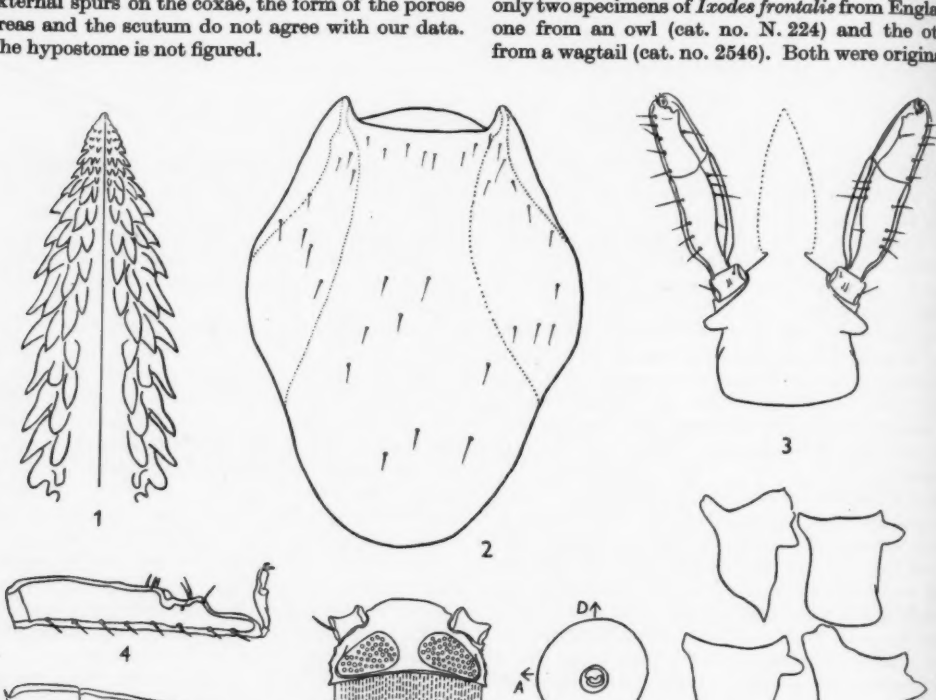

Fig. 1. Hypostome, ventral surface. (Drawn from mounted specimen.)
Fig. 2. Scutum.
Fig. 3. Capitulum from the ventral surface.
Fig. 4. Tarsus of leg I.
Fig. 5. Tarsus of leg IV.
Fig. 6. Basis capituli from dorsal surface.
Fig. 7. Spiracle. *D*, dorsal; *A*, anterior.
Fig. 8. Coxae I–IV, showing internal spurs.

Nuttall *et al.* (1911) base their description of *I. brunneus* on their own previous data for *I. kelloggi* and on Neumann's (1899) figures of *I. frontalis*. The latter were described from specimens collected from *Turdus* in Corsica, three females from *Crex pratensis* (Lower Loire), *Saxicola rubicola* (Paris Museum) and from *Turdus aonalaschkae* Pallasi (Baltimore, Maryland). Since *Ixodes frontalis* has not been recorded from America the tick from *Turdus aonalaschkae* was probably another species. Cooley & Kohls accepted *Ixodes kelloggi* as a synonym of *I. brunneus* Koch, but Bequaert (1946) was uncertain of this. *I. kelloggi* was originally collected from *Habia melanocephala* identified as *I. brunneus*. They do not agree with the typical *brunneus* of Cooley & Kohls, but with the present specimens. A female tick from the same collection (cat. no. 150) found on a dove (*Haplopelia johnstoni* Shelly) from the Mlange mountain, Nyasaland, was also identified as *Ixodes brunneus*. This specimen is *I. daveyi* Nuttall (Nuttall, 1913) and easily distinguished from *I. brunneus* by the shape of the scutum and the horse-shoe shaped anal groove, a character peculiar to *I. lunatus* Neumann, 1907, *I. schillingsi* Neumann, 1901, *I. pilosus* Koch, 1844, and *I. oldi* Nuttall, 1913. *I. daveyi* (Nuttall, 1913) was also recovered from a bird, *Gallirex johnstoni*

Sharpe, on the northern edge of the Rusvenzori, Uganda.

These data support Cooley & Kohls and Bequaert, who expressed the view that *I. brunneus* s.str. is a North American species and the old world ticks, which Nuttall *et al.* referred to as *I. brunneus*, did not belong to the form of North America but to some other species or race. Cooley & Kohls wrote that it is improbable that *brunneus* has been transported by birds across the Atlantic Ocean and it 'follows that records of this species in Europe and Africa are open to scrutiny'. They have disregarded all records of *I. brunneus* originating outside the New World.

My specimens differ from earlier descriptions, with the exception of that of Neumann (1899), in that the teeth are arranged on the hypostome in 4/4 to 3/3 to 2/2 files. Previous writers gave this as 3/3, and Schulze made this a diagnostic character in his key to the German species of ticks (Schulze, in Brohmer, 1944). This dentition formula was also applied to the ticks in the Nuttall collection, but on re-examination I find the formula is 4/4 : 3/3 : 2/2. Nuttall *et al.* indicated that the figure of Neumann might well be distorted owing to mounting; despite this I find that Neumann's figure gives a more correct picture of the dentition of the hypostome. The figures of Neumann and Schulze showed a median, unarmed triangular area on the ventral face of the hypostome, with which the present data agree. It is not particularly evident in the figure by Nuttall *et al.* (Fig. 182) nor in the typical *I. brunneus* (Cooley & Kohls, 1945).

In my specimens the dorso-lateral margins of the basis capituli are neither straight (Nuttall *et al.* 1911) nor gently curved (Schulze, 1933), but rather corrugated and almost black. The cornua are prominent, distinct, postero-lateral prolongations. This does not appear to be so in the figures of Schulze. The auriculae in the Bulgarian specimen are similar to those of *I. daveyi* and form large postero-lateral extensions terminating in a straight edge with two corners. This is also the basic pattern in *I. brunneus*, but in this species they may vary in shape. The specimens of Nuttall and myself have auriculae which are rounded apically and extend beyond the sides of the basis. This is so in both mounted and unmounted specimens. Of previous writers, only Neumann described the cheliceral digit, and except for an additional tooth on the external article my observations on this point agree with his.

The descriptions of the porose areas vary from author to author: thus they are 'large, transverse and of indefinite form' (Nuttall *et al.* 1911), an observation not confirmed by their figure (fig. 182); 'more or less rounded' (Schulze, 1933, fig. 7), while I find them to be subtriangular, with the greatest width mesially and tapering towards the lateral borders. The distance between the pores is less than the greatest breadth of the areae porosae.

Nuttall *et al.* figure the coxal spurs as rounded apically and relatively long; in *I. frontalis* of Schulze they taper to points, and in his *I. frontalis brunneus* they are rounded apically. The coxal spurs of my specimens agree more closely with the latter. Comparison of the British specimens of *I. frontalis* with the North American *I. brunneus* and fig. 79 B in the monograph of Cooley & Kohls convinces me that the coxal spurs are sufficiently distinct to be of diagnostic value.

With the exception of Schulze, most authors agree that the spiracle is broadly rounded, but Schulze figures this as elongate-oval with an excentric macula.

The figures on p. 191 of the Nuttall *et al.* monograph fail to convince me that the authors were figuring *I. frontalis* entirely. The figures of Schulze, based on one specimen, show either a variation from the five British specimens, particularly in form of the auriculae, porose areas, spiracle and coxal spurs, or it is a closely related species.

I. brunneus and *I. frontalis* are possibly two closely related species, each having its characteristic geographical distribution. Very few accurate records of *I. frontalis* on the mainland of Europe are known, but in Britain we now have the following five records of females of this species:

From Owl, Cambridge 1904 (Nuttall collection, N. 224).
From Wagtail, Pulborough, Sussex, 1913 (Nuttall collection, 2546).
From Blackbird, Silwood Park, Sunninghill, 21 August 1950 (PE 412).
From Blackbird, Silwood Park, Sunninghill, 11 August 1950 (PE 404).
From Great Tit, Ashburton, South Devon, 17 October 1950 (PE 539).

All this material is now deposited in the British Museum.

My sincere thanks are due to Mr John Ash for collecting the specimens from Silwood Park, to Dr Glen M. Kohls for the loan of specimens of *I. brunneus* Koch, to Mr E. Browning of the British Museum (Natural History) for permission to examine the tick collection and for assistance in the literature, to Mr T. Hughes for his sustained interest, to Dr B. Dawes for his unfailing encouragement and to Dr P. Tate for reading the manuscript.

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IXODES PASSERICOLA SCHULZE. A DESCRIPTION OF THE HITHERTO UNKNOWN MALE AND LARVA, WITH A RE-DESCRIPTION OF THE FEMALE AND NYMPH

By D. R. ARTHUR

Department of Zoology, King's College, University of London

(With 17 Figures in the Text)

During the course of an investigation into the host-relationship of *Ixodes hexagonus* Leach a large number of birds was examined for its occurrence. These all proved negative for this species, but *I. passericola* Sch. was present on one robin out of 220 examined, on one starling out of eleven, on seventeen great tits out of 163, on twenty blue tits out of 540, and on four nuthatches out of fifty. A detailed report on this aspect is given later.

The female and nymph were originally described by Schulze (1933), but a re-description seems necessary, while the male and larva have hitherto been undescribed. The males were collected from nests.

Female

Body. Unfed specimens measure 2·7 by 1·6 mm., widest at or about spiracular level. Scutum reaching half or more the length of the body; postscutal and marginal body fold nearly smooth and with small hairs. Fully gorged specimens may be as large as 6 by 2·7 mm., and are suboval, usually a little wider back of the middle.

Capitulum (Fig. 1). Length of capitulum 0·33–0·41 mm.; width of basis 0·32–0·40 mm. Basis capituli with the dorsal surface mildly convex, smooth, shining, impunctate and lacking hairs. Posterior margin straight or mildly sinuous, salient. Cornua present as rounded projections. Porose areas suboval or subtriangular, not depressed and lack definite margins; laterally they approach the lateral ridges and posteriorly nearly to the hind margin. Palpi with lateral margins straight, median margin gently curved, widest about midway along its length. Suture between articles 2 and 3 ill-defined, surface wrinkled, shining, impunctate, hairs few and strong, combined length of articles 2 and 3, 0·31–0·34 mm.; width, 0·12–0·15 mm. Venter of basis capituli broad, mildly convex with lateral margins more declivitous, not constricted, posterior edge broadly rounded, salient. Transverse suture, if present, faint. Auriculae absent. Surface even, smooth, impunctate. Palpi with inner faces flattened. Article 1 forms a complete ring.

Hypostome (Fig. 2). Twelve to fourteen lateral serrations to its apex, rounded apically. Sides nearly straight. From the base the teeth arranged thus: 5 rows 2/2, 2 rows 3/3, 2 rows 4/4, 1 row 5/5, 1 row 6/6, the median teeth to the apex thereafter not clearly recognizable. Length 0·21–0·26 mm.

Scutum (Fig. 3). Longer than wide, widest in front of the middle, broadly rounded behind, posterolateral angles convex. Humeral angles broad and rounded. Cervical grooves shallow, faint; lateral carinae indistinct. Surface crazed, especially lateral to cervical grooves; shining with fine punctations. In median area between cervical grooves, slightly elevated and curved downward to lateral margins. Length and width from 0·79 by 0·72 mm. to 0·98 by 0·85 mm.

Legs. Moderate length and breadth, but few bristles, which are found principally on the ventral side. Tarsi (Fig. 4) sharply humped subapically. Length of tarsus I, 0·43 mm., metatarsus I, 0·36 mm.; length of tarsus IV, 0·39 mm., metatarsus IV, 0·35 mm.

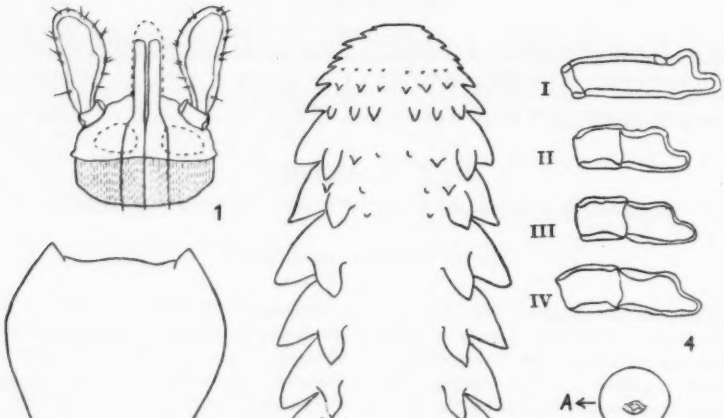
Coxae. Surfaces mildly convex, smooth, faintly punctate. Coxa I with internal spur long and curved. No external spurs. Coxae II to IV lacking both internal and external spurs.

Spiracular plate (Fig. 5). Short oval or sometimes subcircular; longer axis transverse. Macula placed ventral to the centre. Goblets moderate in number, five dorsally, one ventrally. Length of long axis 0·21 mm.

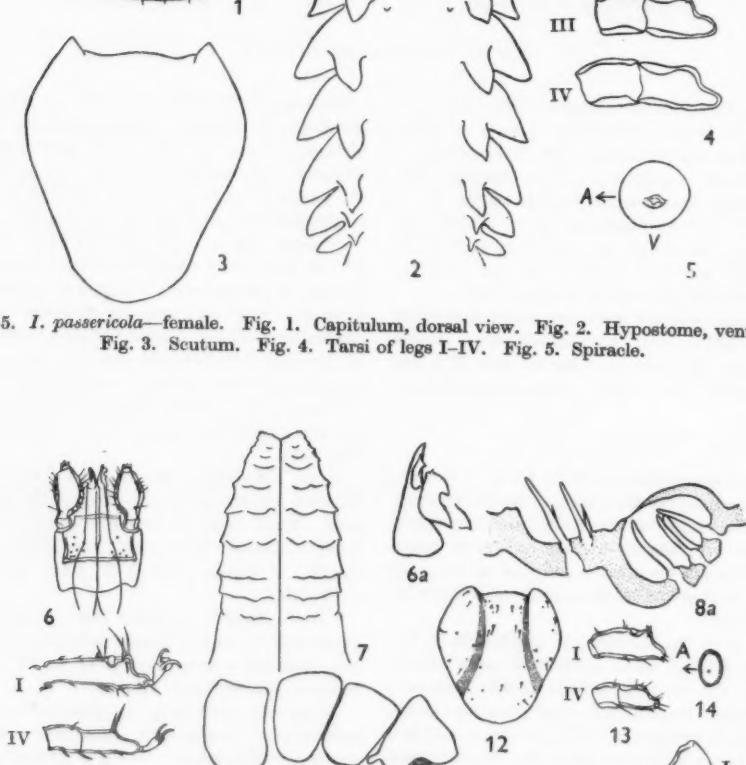
Male

Body. Oval, widest behind the middle. Size 2·4 by 1·6 mm.

Capitulum (Fig. 6). Length 0·27 mm., width of basis 0·21 mm. Basis capituli parallel-sided, sides and posterior border nearly straight, salient. Cornua as faint posterior projections. Surface nearly flat, with few scattered punctations. Palpi short, broad, rounded apically, widest about middle. No indication of sutural line between articles 2 and 3. Bristles, few along lateral borders, long and numerous on mesial face; combined length of articles 2 and 3,



Figs. 1–5. *I. passericola*—female. Fig. 1. Capitulum, dorsal view. Fig. 2. Hypostome, ventral view. Fig. 3. Scutum. Fig. 4. Tarsi of legs I–IV. Fig. 5. Spiracle.



Figs. 6–9. *I. passericola*—male. Fig. 6. Capitulum, dorsal view. Fig. 6*a*. Cheliceral digit. Fig. 7. Hypostome. Fig. 8. Tarsi I and IV. Fig. 8*a*. Haller's organ. Fig. 9. Coxae I–IV.
Figs. 10–15. *I. passericola*—nymph. Fig. 10. Capitulum. Fig. 11. Hypostome. Fig. 12. Scutum. Fig. 13. Tarsi I and IV. Fig. 14. Spiracle. Fig. 15. Coxae I–IV. Fig. 16. Capitulum and scutum of the larvae of *I. passericola*. Fig. 17. Hypostome of the larva of *I. passericola*.

0·20 mm.; width 0·10 mm. Basis in ventral view broad, long and nearly flat. Broadly rounded behind, salient at the sides. Auriculae absent.

Hypostome (Fig. 7). Length 0·13 mm. Apically notched. Thirteen to fifteen lateral projections; seven to nine transverse rows of crenulations, with crenulations nearly meeting in midline.

Scutum. About equally convex in anterior and posterior areas. Surface smooth. Punctations in median area fine and moderate in number; those in lateral and posterior areas large. Hairs sparse and fine, arising from very small punctations. Post-humeral areas of surface mildly tumescent, limits of pseudoscutum only faintly indicated. Cervical grooves shallow and faint. Length 2·08 mm.; width 1·4 mm.

Legs (Figs. 8, 9). Essentially as in female. Length of tarsus I, 0·35 mm., metatarsus I, 0·29 mm.; length of tarsus IV, 0·30 mm., metatarsus IV, 0·22 mm.

Spiracular plate. Circular, with surface mildly convex; macula a little anterior and ventral to the centre; transverse axis measures 0·18 mm.

Plates. Median plate longer than broad, as 5·2 to 4·2, narrower in front; anal plate shorter than median :: 4·2 : 5·2, with lateral margins gently convex; adanals with parallel sides. Surfaces of plates punctate and with fine short hairs.

Genital aperture. Opposite second intercoxal space.

Nymph (Figs. 10–15)

Capitulum (Fig. 10). Length from dorsal ridge to apex of hypostome, 0·19 mm.; width of basis (across cornua), 0·20 mm. Nearly level, smooth, shining, impunctate; posterior margin sinuous, salient. Cornua small, rounded. Palpi with lateral margins straight and median margins curved; widest in front of the middle. Combined length of articles 2 and 3, 0·19 mm. Sutural line between articles 2 and 3 absent. Surface slightly wrinkled; with few long bristles. Palpal article 1 rounded. Ventral view of capitulum broad, rounded and salient behind. Auriculae as mild humps. Palpi concave on their inner surfaces.

Hypostome (Fig. 11). Curved on the sides, apex rounded. Denticles arranged from the base in five rows 2/2, two rows 3/3, one row 4/4. Apically median teeth not clearly identifiable, but ten or eleven lateral rows. Teeth in lateral and median files about equal in size. Length from 0·11 to 0·13 mm.

Scutum (Fig. 12). Length 0·42–0·44 mm., width 0·40–0·41 mm. Rounded posteriorly, widest in front of the middle. Lateral carinae absent. Surface crazed, punctations sparse, scapulae rounded.

Legs. Haller's organ nearly midway from the distal end. Length of tarsus I, 0·22 mm., metatarsus I, 0·17 mm.; length of tarsus IV, 0·20 mm., metatarsus IV, 0·15 mm.

Larva (Figs. 16, 17)

Capitulum (Fig. 16). Length from dorsal ridge to hypostomal apex 0·10 mm.; width of basis 0·11 mm. Posterior margin straight, cornua small, rounded. Palpi with lateral margins straight, mesial borders convex; widest in front of the middle. Combined length of articles 2 and 3, 0·10 mm. Sutural line between articles 2 and 3 absent. Ventrally capitulum broad, with greatest width on level with origin of palps, thereafter sides converging to a broad posterior convexity.

Hypostome (Fig. 17). Apex rounded, sides curved. Denticles from apex to base arranged as two rows of 3/3, five rows of 2/2, those in median and lateral files about equal in size. Length about 0·06 mm.

Scutum. Length 0·24 mm., width 0·27 mm. Widest in front of the middle. Cervical grooves and lateral carinae faint. Punctations sparse and faint. Scapulae rounded.

Previously *I. passericola* had been collected from a starling in Mecklenburg, Germany, by Schulze (in Brohmer, 1944). It has also been recorded as *I. hexagonus* from a blue tit in Richmond, Surrey, by Thompson (1937).

Reference to Tables 1 and 2 shows that only five species of birds out of forty-six investigated and forty-three of 1858 individuals examined (or 2·3 %) were infested with one or more developmental stages of *I. passericola*. The ticks were usually found around the eyes and on the neck, but a fully fed female tick was collected from the coccyx of a starling.

Without knowing the seasonal occurrence of *I. passericola* on birds it is difficult to assess the host range, particularly as the trapping of birds is beset by practical difficulties. Birds can be caught with ease in the winter (October to March) but this is difficult in the summer (April to September). Accordingly, winter catches are high and summer catches low. To overcome this, juveniles and nestlings were also examined in the summer, but the numbers of each species available were inadequate for comparison. I have also been unable to ascertain whether tick populations on the adults and immature birds differ significantly. Consequently, summer and winter samples may not be comparable.

Only one female tick was collected from 220 robins, and it is thought that the numbers of birds investigated are adequate and sufficiently well distributed over the year to give a true picture of the occurrence of the tick on this host. Probably the robin is an accidental host. The numbers of starlings examined, however, are inadequate to be certain on this point. The three other infested species are the British nuthatch, the British blue tit and the British great tit. These are confined to the British Isles and their migrations are generally only local movements (Witherby, Jourdain, Ticehurst & Tucker, 1938).

Table 1. *Number of birds parasitized by* Ixodes passericola *in Silwood Park* (1949–50)

	Robin		Starling		Nuthatch		Great tit		Blue tit	
	P*	E	P	E	P	E	P	E	P	E
1949										
Jan.	0	17	—	—	—	—	0	3	0	144
Feb.	0	15	—	—	0	2	0	15	3	173
Mar.	0	11	—	—	0	1	1	22	1	45
Apr.	—	—	—	—	—	—	1	1	—	—
May	0	13	—	—	0	1	0	8	—	—
June	—	—	—	—	—	—	—	—	—	—
July	—	—	—	—	—	—	—	—	—	—
Aug.	0	7	—	—	1	4	0	9	0	8
Sept.	0	2	—	—	1	1	—	—	0	7
Oct.	0	9	—	—	0	4	1	13	0	15
Nov.	0	6	—	—	0	3	6	14	0	12
Dec.	0	12	0	2	0	7	1	12	2	23
1950										
Jan.	0	26	0	1	0	6	3	15	4	24
Feb.	0	29	—	—	0	3	1	14	1	29
Mar.	0	15	0	1	0	5	0	10	6	30
Apr.	0	2	—	—	—	—	1	3	0	2
May	1	11	—	—	—	—	—	—	—	—
June	0	10	1	7	1	1	0	10	0	21
July	0	15	—	—	0	2	0	2	0	2
Aug.	0	20	—	—	1	10	2	12	3	5

* P, number of birds parasitized by *I. passericola*; E, number of birds examined.

We cannot, however, at this stage, make any claim that *I. passericola* is specific to these three hosts, for the numbers of other bird species investigated are considerably fewer.

As expected, larvae were more numerous than nymphs on the hosts, and nymphs more numerous than females. The latter rarely exceeded one per host, but we did find two female ticks once. Schulze (1932) and Shcheglova (1939) stated that adult females are rare on the majority of wild birds, probably because of self de-ticking. Milne (1949) confirmed this and showed that birds never have ticks attached within beak length. Males of *I. passericola* were collected only from the nests of birds. Their absence from the birds themselves coupled with the hypostomal structure suggests that they resemble *I. hexagonus* in this respect.

This work would not have been possible were it not for the very generous assistance of Mr John Ash, who supplied the material and kindly permitted me to use his collection data in compiling this information. Mr E. Browning of the British Museum (Natural History) also gave me considerable assistance in checking my identification, and I am grateful to him for this.

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Table 2. *Species and number of birds examined and number parasitized by* Ixodes passericola

(Examined in collaboration with Mr John Ash; all from Silwood Park, Berks.)

		No. examined	No. parasitized
Blue tit	*Parus caeruleus obscurus*	540	20
Great tit	*P. major newtoni*	163	17
Marsh tit	*P. palustris dresseri*	13	0
Continental blue tit	*P. caeruleus caeruleus*	7	0
Coal tit	*P. ater brittanicus*	13	0
Songthrush	*Turdus ericetorum ericetorum*	77	0
Blackbird	*T. merula merula*	150	0
Redwing	*T. musicus musicus*	2	0
Missel thrush	*T. viscivorus viscivorus*	16	0
Nuthatch	*Sitta europaea affinis*	50	4
Chaffinch	*Fringilla coelebs*	322	0
Robin	*Erithacus rubecula melophilus*	220	1
Jackdaw	*Corvus monedula spermologus*	19	0
Rook	*C. frugilegus frugilegus*	1	0
Carrion crow	*C. corone corone*	5	0
Hedge sparrow	*Prunella modularis occidentalis*	15	0
Bullfinch	*Pyrrhula pyrrhula nesa*	3	0
Jay	*Garrulus glandarius rufitergum*	14	0
Starling	*Sturnus vulgaris vulgaris*	11	1
Partridge	*Perdix perdix perdix*	1	0
Tawny owl	*Strix aluco sylvatica*	1	0
Moorhen	*Gallinula chloropus chloropus*	1	0
Green woodpecker	*Picus viridis virescens*	4	0
Long-tailed tit	*Aegithalos caudatus roseus*	1	0
Wood warbler	*Phylloscopus sibilatrix sibilatrix*	4	0
Chiff chaff	*P. collybita collybita*	5	0
Willow warbler	*P. trochilus trochilus*	15	0
Mallard	*Anas platyrhyncha platyrhyncha*	3	0
Spotted flycatcher	*Muscicapa muscicapa striata*	16	0
Tree creeper	*Certhia familiaris brittanica*	7	0
Spotted woodpecker	*Cryobates major anglicus*	2	0
Greenfinch	*Chloris chloris chloris*	1	0
Wren	*Troglodytes troglodytes troglodytes*	24	0
Tree pipit	*Anthus anthus trivalis*	43	0
Grey wagtail	*Motacilla motacilla cinerea*	10	0
Wood lark	*Lullula lullula arborea*	20	0
Little owl	*Carine noctua mira*	6	0
Whitethroat	*Sylvia communis communis*	5	0
Lapwing	*Vanellus vanellus vanellus*	5	0
Nightingale	*Luscinda megarhyncha megarhyncha*	6	0
Goldfinch	*Carduelis carduelis brittanica*	1	0
Woodcock	*Scolopax rusticola rusticola*	1	0
Cuckoo	*Cuculus canorus canorus*	1	0
Wood pigeon	*Columba palumbus palumbus*	2	0
Magpie	*Pica pica pica*	2	0
Kestrel	*Falco tinnunculus tinnunculus*	3	0

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